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AN ATTEMPT TO STUDY THE MECHANISM OF AMYLASE ACTION BY THE USE OF C^{14} LABELED STARCH

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Akabori et al. recently reported [1] that crystalline Taka-amylase A preparation obtained from a synthetic culture medium of *Asp. oryzae* contained as little sugar as 0.25% (calculated as glucose), while another crystalline preparation obtained from "Taka-diastase Sankyo" contained 2.5% sugar. Such a large discrepancy in sugar content between two preparations might suggest that the mechanism of amylase action is a primary transfer of glucosidic residues from starch molecules to amylase proteins, most probably to the hydroxyl groups of the protein, followed by the hydrolysis of the glucoside bonds.

If the assumption were correct, the sugar group which previously occupied the active centre of the enzyme molecule should be replaced by some sugar group liberated from starch during the amylase action. The replacement of the sugar group could easily be detected by the use of uniformly C^{14} labeled starch as the substrate.

The present paper deals with experiments carried out for the purpose of verifying the above mentioned assumption. The results obtained are, however, rather contradictory to the assumption and further experiments are required to come to a final conclusion.

METHODS AND MATERIALS

Radioactive starch: Prepared photosynthetically by the method of Hassid[2] using leaves of Michaelmas daisy. Specific activity of this material was 156,000 c.p.m./mg.

Crystalline Taka-amylase A: Isolated from the crude Taka-diastase and recrystallized several times.

Measurement of radioactivity incorporated into the crystalline Taka-amylase A: Radioactive starch was digested by the crystalline Taka-amylase A under various experimental conditions and then, usually after 1 hr., the enzyme was isolated from the reaction medium in a crystalline state after several purifying procedure. Final crystallization was achieved by the dropwise addition of cold acetone into the enzyme solution, in M/50 Ca acetate. The crystals were collected by centrifugation, washed with 50%

acetone, and dissolved in the M/50 Ca acetate solution. An aliquot of this solution was taken and amylase protein was determined colorimetrically with the Folin reagent[3]. Another aliquot was dried on a stainless steel dish under an infra red lamp and the radioactivity was measured with the Geiger-Müller counter. Specific radioactivity was expressed as c.p.m. per mg of crystalline Taka-amylase A. The quantities of glucose residue incorporated into 1 M of Taka-amylase A were calculated from the above observed value and the spcific activity of the substrate starch assuming the molecular weight of Taka-amylase A to be 47,000[4].

EXPERIMENTAL

1. Incorporation of C^{14} into the crystalline Taka-amylase A during the degradation of C^{14}-labeled starch.

4.2 cc of the crystalline Taka-amylase A solution in M/50 Ca acetate (containing 475 mg of enzyme) and 10 cc of M/30 Ca acetate-acetic acid buffer (pH 5.2) were mixed and into this solution 4 cc of radioactive starch solution (containing 201 mg of starch) was added dropwise while shaking continuously. After incubation at 30°C for 1 hr., 2 vol. of acetone were added to this reaction mixture. Precipitated amylase was collected, washed with 60% acetone, dissolved in M/50 Ca acetate solution and dialyzed in an ice box against the M/50 Ca acetate solution to remove concomitant low molecular products. The outer solution was renewed daily. After 4 days, the enzyme was precipitated with acetone, washed, redissolved in M/50 Ca acetate solution, and crystallized from this solution by the dropwise addition of acetone (first crystallization). The crystals were dissolved in M/50 Ca acetate solution and 5.7 g of $(NH_4)_2SO_4$ solution of the same concentration, redissolved in M/50 Ca acetate solution, dialyzed as before, then precipitated with acetone, redissolved, and finally recrystallized (Second crystallization). The enzyme was recrystallized once more in the same way (Third crystallization). The specific activity of these crystals and the mother liquor of the final crystallization are shown in Table 1.

Table 1. Incorporation of C^{14} into the crystalline Taka-amylase A during the degradation of C^{14}-labeled starch

	Specific activity c.p.m./mg. Taka-amylase A	Total activity (c.p.m.)	M of glucose residue incorporated into 1 M of Taka-amylase A
Once crystallized amylase	117.3	55,700	0.217
Twice ,, ,,	52.5	24,900	0.098
Thrice ,, ,,	19.5	9,300	0.036
Mother liquor of third crystal	18.8		

As shown in Table 1, a considerable amount of radioactivity was found in the recrystallized Taka-amylase A and could not be removed by various purification procedures, i.e. dialysis, acetone precipitation, $(NH_4)_2SO_4$ pre-

cipitation, or recrystallization, though it decreases gradually. Furthermore the specific activity of the thrice crystallized amylase and the mother liquor were almost equal. These results hardly show that the observed incorporation of radioactivity is due to mere contamination.

In the experiment described above, the total activity of the substrate starch was 3135,000 c.p.m. and that incorporated into the first crystals was 55,700 c.p.m., therefore 1.7% of the original substrate was incorporated. In another experiment shown in Table 2, in which the amount of enzyme used was smaller while the amount of substrate was equal to that used in the experiment shown in Table 1, the specific activity of the isolated crystals was greater. But the proportion of incorporated radioactivity to that of the substrate starch was 1.84%, apparently equal to the value obtained in the first experiment described above.

2. *Nature of the radioactive sugars incorporated*:

(1) Behavior under various treatments.

As already described, the radioactive sugars incorporated could be separated only very gradually from the enzyme protein by the usual purification procedure, e.g., dialysis, acetone precipitation, $(NH_4)_2SO_4$ precipitation, or recrystallization. (Therefore the binding of the incorporated sugar to the protein part seems to be relatively stable.)

(2) Behavior under thermal denaturation of the protein.

5 cc of the Taka-amylase A solutin in M/500 Ca acetate (containing 2.2 mg of protein) was boiled for 3 min. and the precipitated protein was centrifuged, washed with water, and its radioactivity was measured.

Table 2.

	c.p.m.
Radioactivity of the original crystals (containing 2.2 mg. of protein)	2490
Radioactivity of denatured protein	0

As shown in Table 2, incorporated radioactivity was quantitatively removed from the protein part by thermal denaturation.

(3) Behavior under paper electrophoresis.

A sample or radioactive Taka-amylase A was subjected to paper electrophoresis and the behaviors of radioactivity and protein were examined. After being developed electrophoretically with M/20 veronal buffer (pH= 8.0), the filter paper was air-dried and protein was detected by ninhydrin reagent and by amidschwarz 10 B reagent, or measured after elution with water, by the optical density at 280 mμ. Amylase activity was detected by the usual iodine-starch reaction. For the radioactivity measurement, the paper was cut into a number of pieces of equal size, dried and counted. Results shown in Figs. 1 and 2 indicate that the major part of the radioactivity was separated from the protein part but a small quantity still remained in the enzyme protein.

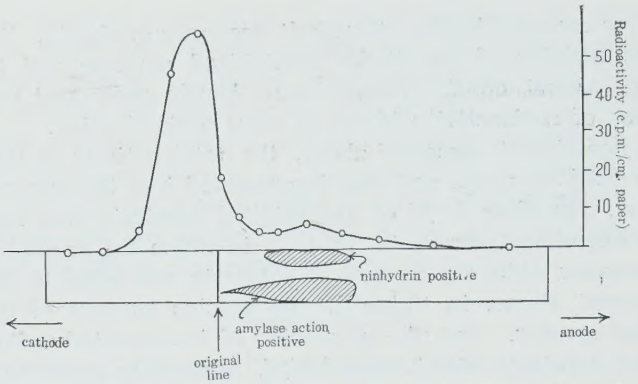

Fig. 1. Paperelectrophoresis of radioactive Taka-amylase A

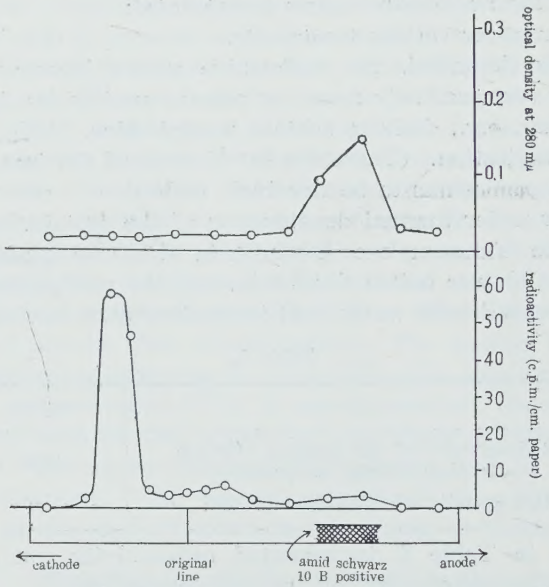

Fig. 2. Paperelectrophoresis of radioactive Taka-amylase A

(4) Tests for incorporated sugars by paperchromatography.

As already described, radioactivity incorporated into Taka-amylase A could be separated from the protein moiety by thermal denaturation and released quantitatively into the supernatant solution. This radioactive supernatant freed from the protein part was concentrated on a water bath and chromatographed with carrier glucose, maltose, isomaltose, and pannose.

After the three developments with n-butanol-pyridine-water (3:2:1.5) carrier sugars were detected with ammoniacal $AgNO_3$, and radioactivity was measured directly on the paper (Fig. 3). Activity was observed only near the starting point, and none of the spots of the added sugars showed radioactivity. Fig. 3 also shows that no new spots other than those of

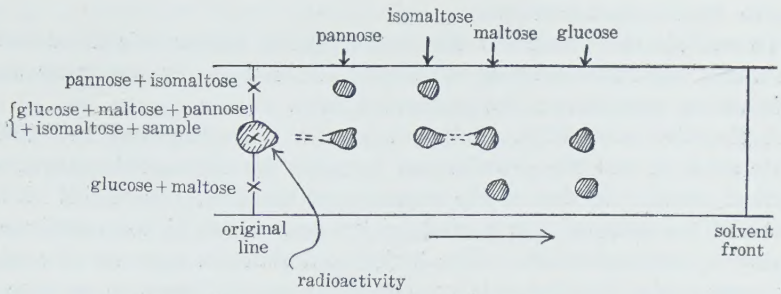

Fig. 3. Paperchromatogram of the supernatant of the boiled radioactive Taka-amylase A solution

added sugars were detected on the paper.

Considering the possibility that the incorporated radioactive matter might be a high molecular substance combined with peptide and therefore unable to move on the paper, the detection of both radioactivity and ninhydrin positive substances was attempted in the following manner. Large amounts of non-labeled Taka-amylase A were denatured by boiling, the supernatant was concentrated and spotted with the radioactive sample prepared in the same manner, developed with n-butanol-acetic acid-water (4:1:4). Amino acids or pepides were detected with the ninhydrin. Radioactivity was measured on each piece of the cut paper. As shown in Fig. 4,

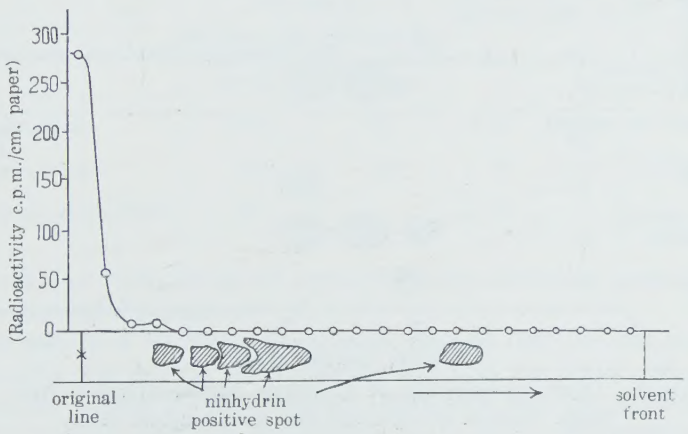

Fig. 4. Paperchromatogram of the supernatant of the boiled radioactive Taka-amylase A solution

five ninhydrin positive spots were detected but all the radioactivity remained near the starting point and none could be detected on the other spots. Therefore it can be concluded that Taka-amylase A releases several amino acids or peptides and incorporated radioactive sugars as a result of thermal denaturation, but radioactive sugar is released in a free state, not combined with protein. Therefore the incorporated sugars seem to be polysaccharides

of more than 4 glucose units.

To exclude the possibility that incorporated sugars might be derived from minor impurities such as xylosane contained in the substrate starch, the following experiment was performed next.

Radioactive crystalline Taka-amylase A was dissolved in M/50 Ca acetate solution and the protein was removed by thermal denaturation as described above. To 4 cc of the supernatant solution, 1 cc of 5 N HCl was added and the mixture was heated for 2.5 hrs. at 100°C to hydrolyze the radioactive polysaccharides. The hydrolyzed solution was centrifuged and clear supernatant solution was vacuum dried several times to remove HCl completely. The residue thus obtained was dissolved in a small amount of water and cochromatographed with carrier glucose, using n-butanol-pyridine-water (3:2:1.5) as the solvent and ammoniacal $AgNO_3$ as the color reagent.

Fig. 5 clearly shows that almost all the radioactivity was observed on the glucose spot.

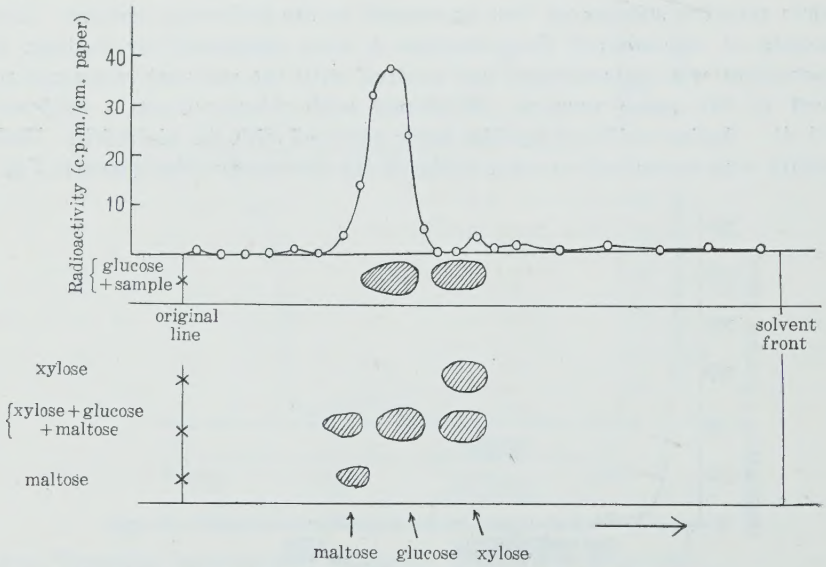

Fig. 5. Paperchromatogram of hydrolyzed sample of radioactive sugar fraction incorporated into Taka-amylase A

Another spot on the paperchromatogram which corresponds to xylose showed no radioactivity. This pentose must have been in the original Taka-amylase A crystals and released by thermal denaturation and hydrolysis.

3. Comparison between the incorporation of C^{14} from the labeled starch and from the degradation products of labeled starch.

The above experiments showed that radioactive sugar were incorporated into Taka-amylase A when this enzyme acted on radioactive starch. But

there is no indication as to whether this phenomenon has a direct relationship with the mechanism of amylase action or whether this is merely due to secondary absorption of the degradation products into the protein. In order to determine which of these two alternatives is correct, the following experiments were performed. 19.8 mg of Radioactive Starch were degraded by crystalline Taka-amylase A under conditions similar to those in Experiment 1. Then the resultant solution was boiled and the supernatant solution, which contained the degradation products and the incorporated radioactive sugars was concentrated. 32 mg of crystalline Taka-amylase A were incubated with the above concentrated solution at 30°C for 1 hr. and from this reaction mixture, the enzyme was reisolated and purified to the crystalline state. Its specific activity was compared with that of the control where 19.8 mg of radioactive starch was directly degraded by 51.8 mg of crystalline Taka-amylase A.

Table 3 shows the results which indicate that the incorporation of the radioactive sugars was greater when amylase acted on the starch than when it acted on the degradation products of the starch.

Table 3. Incorporation of C^{14} from the labeled starch and from the degradation products of labeled starch

	Specific activity (c.p.m./mg. Taka-amylase A.		M of glucose residue incorporated per 1 M of Taka-amylase A		Total activity incorporated (c.p.m.)	
	(A) From the labeled starch	(B) From the degradation products	(A)	(B)	(A)	(B)
Once crystallized	1099	217	2.04	0.40	56,900	6,900
Twice crystallized	962	102	1.79	0.19	49,800	3,300
Three times crystallized	601		1.12			

4. Decrease of radioactivity in crystalline Taka-amylase A during its action on non-labeled starch.

The results of the above experiment showed that the incorporation of radioactivity was greater when amylase acted on the starch and could not be explained merely by the secondary absorption into the protein. Therefore it is possible that this phenomenon has a direct relationship with the mechanism of amylase action and that the incorporation occurs at the active center of the enzyme. If so, this incorporation is obligatory for the action of the enzyme, and incorporated sugars must be exchanged with the non-labeled sugars when the subsequent action is on non-labeled starch. This was tested as follows.

Non-labeled starch was degraded by radioactive crystalline Taka-amylase A and the enzyme crystals were reisolated and the specific activity was determined. As a control experiment, the same radioactive crystalline

enzyme was incubated with the degradation products of the non-labeled starch and the specific activity of the reisolated enzyme crystals was compared with that of the experimental one (Table 4).

Table 4. Decline of radioactivity in crystalline Taka-amylase A during its action on non-labeled starch

	Specific activity of crystals (c.p.m./mg)	% of decrease
Initial	36.9	
After the action on non-labeled starch	12.0	67.6
After the treatment with the degradation products of non-labeled starch	12.6	65.9

Contrary to expectations, the degree of the decrease of radioactivity in both cases was about the same (32–34%) and a comparison with the results of Tables 1 and 2, shows that it is of about the same order as that produced by recrystallization.

5. Relationship between the incorporated sugars and enzyme activity.

If incorporated sugars have a direct relationship with the enzyme activity, it might be supposed that the protein part freed from incorporated sugars shows the decreased activity or, at least, an induction period at the very early stage of the enzyme action.

As shown in Experiment 2, most of the incorporated radioactivity is freed from the protein part by paper electrophoresis. The enzyme protein thus obtained was eluted from the paper and its enzymatic activity was compared with that before the electrophoresis. The substrate, 50 cc of 1.2% starch solution in M/25 phosphate, was degraded by the both enzyme solutions containing 11.0 γ of protein, and reducing sugars liberated were determined at successive intervals. The difference observed between these two samples was within the margin of error of the analytical method employed.

DISCUSSION

1. Using radioactive starch as substrate, it was shown that when Taka-amylase A hydrolyzed the starch a portion of the degradation products combined with the protein and could not be removed by the usual purifying procedure or recrystallization. Moreover this incorporation was far greater when this enzyme acted on the starch than when acted on its decomposition products. So this cannot be explained merely by contamination. Furthermore, the total amount of incorporated sugars was about 2% of the substrate starch and when the amount of Taka-amylase A employed was small, about 1 to 2 mol. glucose residue was incorporated into one mol. of Taka-amylase A.

Since in the experiment described above very small amounts of starch were used as substrate for the large amount of enzyme and possibly only

a small fraction of the enzyme performed the catalytic action, it is thought that when a large amount of the substrate starch is hydrolyzed by a small amount of enzyme this incorporation might be greater.

2. From the results of Experiment 4 in which the decline of radioactivity in Taka-amylase A was not demonstrated during the action on non-labeled starch, it may be concluded that incorporated sugar is not excahnged with additional substrate sugars during further enzymatic action, therefore has no relation with the mechanism of enzyme action. This conclusion seems to be further supported by the fact that the protein part almost completly freed from incorporated sugars by the electrophoresis could show the same enzymatic activity as that of untreated amylase.

However, as already described, in this experiment very small amounts of substrate were used compared with the enzyme amount and only the amylase molecule, into which no radioactivity was incorporated previously, may have acted in the next experiment. This could explain the result of Experiment 4. Furthermore the detection of the difference between the activity of the two enzyme samples, before and after paper electrophoresis, cannot be regarded as evidence supporting the above conclusion, in view of the fact that the radioactivity was not completely removed by this treatment and no test were performed with regard to the length of the induction period at the very early stage of enzyme action. These problems necessitate further investigation.

3. Experiment 2 demonstrated that incorporated radioactivity resided only in the spot of carrier glucose and not in the spot of xylose which was released from the Taka-amylase A by thermal denaturation and subsequent acid hydrolysis. These results together with other properties of radioactive sugars, show that observed incorporation of radioactive sugars has no direct relationship with the sugar moiety of Taka-amylase A which is already reported.

SUMMARY

Radioactive starch was digested with crystalline Taka-amylase A and the incorporation of the radioactivity into the enzyme crystal was investigated under various conditions.

The incorporated radioactive sugar seems to be polysaccharide in nature and, though this radioactive sugar could not be removed by various purifying procedures, it was released from the protein part by boiling or paper-electrophoresis.

Possible implications of this incorporation was discussed.

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THE ELECTRON MICROSCOPIC INVESTIGATION ON CLASSIFICATION OF CONIDIA OF THE GENUS ASPERGILLI*

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The nature of the conidial walls and conidiophore walls of *Aspergillus oryzae* and *Aspergillus sojae*,[1] which are widely used in Japan for manufacturing alcohol, sake, soy sauce, and soy paste, are the essential characteristics for distinguishing these species from others. Similarly, the nature of conidial walls of Kuro-Koji molds,[2] which are widely used for manufacturing a kind of distilled wine from sweet potatoes in the southern districts of Japan, is one of the essential characteristics for distinguishing it from ordinary *Aspergillus niger*.

It was proposed by K. Sakaguchi and the present author (1949, 1953, 1954)[3][4][5][6][7] that the electron microscope is an effective and indispensable apparatus for the determination of these characteristics.

As it is well known, the nature of the mature conidial walls of Aspergilli differ from speies to species. This characteristic as well as the size, shape, and color of conidia[8][9][10][11] are the essential characters used in the taxonomy of this genus. But in some cases, there is no clear dividing line among the different species when observed with an optical microscope and we hesitate over the choice of a suitable descriptive term, or researchers use different terms for the same species.

The author succeeded in showing electron micrographs of conidia of many species of Aspergilli, and to clarify the micro-morphological characters, especially the nature of conidal walls. In order to systematize the descriptive terms for the conidial walls, the author classified the micro-morphological characteristics of the conidia.

MATERIALS AND METHODS

One hundred forty three typical strains of Aspergilli, obtained from Dept. Agr. Chem. Univ. of Tokyo. Centraalbureau voor Schimmelcultures, and from my own collection, were used in this study.

Conidia obtained from Czapek's solution agar cultures incubated for 21 day at 30°C were put on a clean 200 mesh copper screen on which a thin Formvar "(Vinyl acetate formaldehyde polymer)" film[12] or Collodion-SiO film had previously been mounted and dried.

* This was read at the General Meeting to the Agricultural Chemical Society of Japan held in Sapporo, in August, 1953.

The specimen were examined with a HITACHI type HU-4 or NIHON-DENSHI type DA-4, electron microscope.

The electron micrographs were all taken at an electronic magnification of 2500× or 3000× and then enlarged.

RESULTS AND DISCUSSION

1. Contraction of the specimen by the electron beam:

All the specimen were observed under 1 c<20 mA. Under 1 c=30 mA, some contraction of the specimen due to the electron beam was observed as shown in Fig. 2, a photograph of *Asp. tamarii* as an example. The surfaces are covered by conspicuous processes. Fig. 1 was taken at 1 c<20 mA and Fig. 2 at 1 c=30 mA.

2. A "connective" or "disjunctor" a bridge between conidia in the chain:

Fig. 3, a photograph of *Asp. usamii*, one of the useful black Aspergilli, is an example of conidia during their early stage of growth. They lie down in a thin membraneous tube.

When mature they assume the size and shape characteristic of the species as shown Fig. 4. The "connective" or "disjunctor", the bridge between conidia in the chain, often cited in descriptive literature[10][13] consists of portions of the outer wall of the thin membraneous tube.

3. Nature of the conidial surface:

Electron micrographs of typical species of Aspergilli are shown in Fig. 5 to Fig. 40 in order to clarify the micro-morphological characters, especially the nature of the surfaces of mature conidial-walls. Many studies have been made of the characteristic size, shape, and surface of mature conidia of Aspergilli from the taxonomical point of view. But in some cases the differences between species are so slight when observed with an optical microscope, that it is difficult to choose a suitable descriptive term and many researchers use the various terms for the same species. In order to systematize the descriptive terms for the conidial walls the author classified the micromorphological characteristics of conidia as shown in Table 1; i.e. the nature of conidial walls are (1) aculeate (Fig. 5 and 6), (2) conspicuously echinulate (Fig. 1, 7, 8, 9, 10, 11 and 28), (3) echinulate (Fig. 12, 13, 14, 15, 16 and 17), (4) delicately echinulate (Fig. 18, 19, 20, 21, 22, 23, 24, 25, 26 and 27), (5) loosely rough (Fig. 4, 29 and 30), (6) spinulose (Fig. 31, 32 and 33) and (7) smooth (Fig. 34, 35, 36, 37, 38, 39 and 40), and the shape of conidia are (I) globose (II) subglobose and (III) elliptical.

4. Differences between optical microscopy and electron microscopy:

a. The conidia of both *Asp. candidus* Link and *Asp. niger mut. cinnamomeus* (Schieman) Thom and Raper (=*Asp. cinnamomeus* Schieman) are globose and have smooth surfaces when observed with an optical microscope. But with the electron microscope, the former is quite smooth (Fig. 34) while the latter is not (Fig. 29).

b. Conidial walls of *Asp. melleus* Yukawa are recorded as smooth

Table 1. Classification of the nature of the conidial walls of Aspergilli.

Nature of conidial walls \ Shape of conidia	I. globose	II. subglobose	III. elliptical
1. aculeate	*A. japonicus* Fig. 5.		*A. aculeatus*[7] Fig. 6.
2. conspicuously echinulate	*A. carbonarius* Fig. 7. *A. tamarii* Fig. 1. *A. niger* Fig. 8. *A. phoenicis* Fig. 9. *A. pulverulentus* *A. parasiticus* Fig. 10. *A. variecolor* Fig. 11. *A. niger mut. schiemanni* Fig. 28.		
3. echinulate	*A. terricola* Fig. 12. *A. fumigatus* Fig. 13. *A. ustus* Fig. 16. *A. sojae*[3] Fig. 17. *A. aureus*	*A. itaconicus* Fig. 14.	*A. ostianus* Fig. 15.
4. delicately echinulate	*A. ochraceus* Fig. 18. *A. nidulans* Fig. 19. *A. rugulosus* Fig. 20. *A. oryzae* Fig. 21. *A. flavus* Fig. 22.	*A. awamori* Fig. 23. *A. wentii* Fig. 24. *A. elegans* Fig. 25. *A. medius* Fig. 26. *A. restrictus* Fig. 27. *A. cellulosae*	*A. gracilis*
5. loosely rough	*A. usamii*[4] Fig. 4. *A. inuii*[4] *A. niger mut. cinnamomeus* Fig. 29.	*A. melleus* Fig. 30.	
6. spinulose	*A. sydowi* Fig. 31. *A. versicolor* Fig. 32.		*A. repens* Fig. 33.
7. smooth	*A. candidus* Fig. 34.	*A. sulphureus* Fig. 35. *A. flavipes* Fig. 36. *A. terrus* Fig. 37.	*A. clavatus* Fig. 38. *A. giganteus* Fig. 39. *A. alliaceus* Fig. 40.

when observed through an microscope, but with electron microscope they are not (Fig. 30). The projections of conidia are within the limit of the resolution power of optical microscopes.

c. The conidia of both *Asp. fumigatus* Fresenius and *Asp. japonicus* Saito are recorded as equally echinulate in many studies. But now it is clear that they are not the same. The author proposes that conidia of *Asp. fumigatus* is echinulate (Fig. 13) and of *Asp. japonicus* is aculeate (Fig. 5).

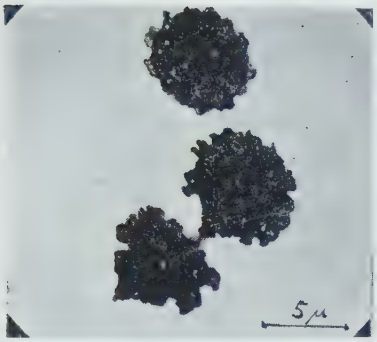


Fig. 1. *A. tamarii* (I_c<20$_m$A)

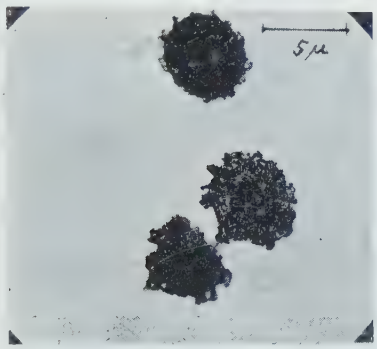


Fig. 2. *A. tamarii* (I_c=30$_m$A)

Fig. 3. *A. usamii* (young stage)

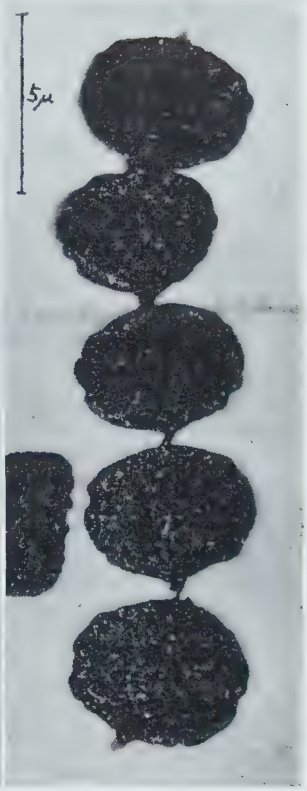


Fig. 4. *A. usamii* (mature)

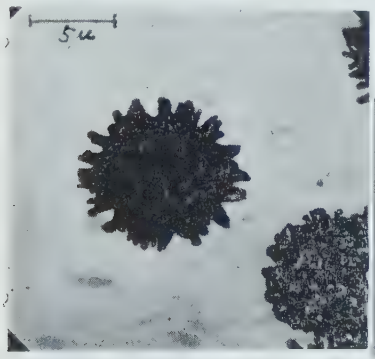


Fig. 7. *A. carbonarius*

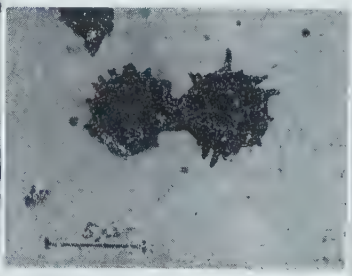


Fig. 5. *A. japonicus*

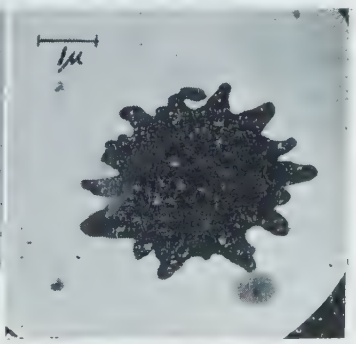


Fig. 8. *A. niger*

Fig. 6. *A. aculeatus*

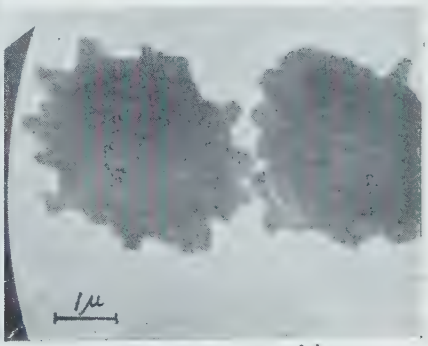


Fig. 9. *A. phoenicis*

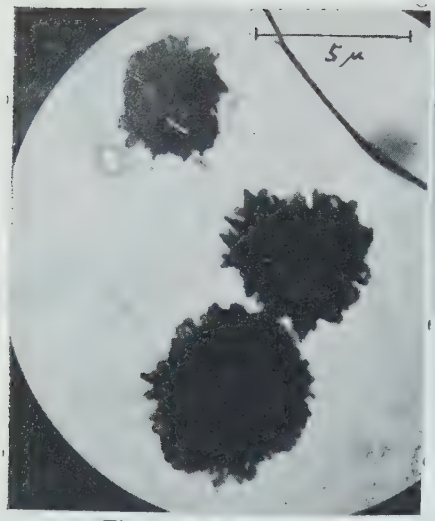
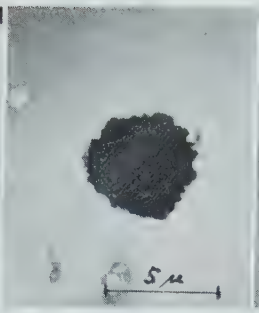


Fig. 10. *A. parasiticus*



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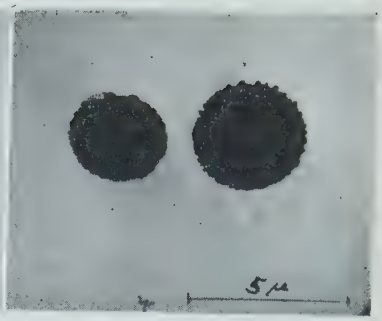

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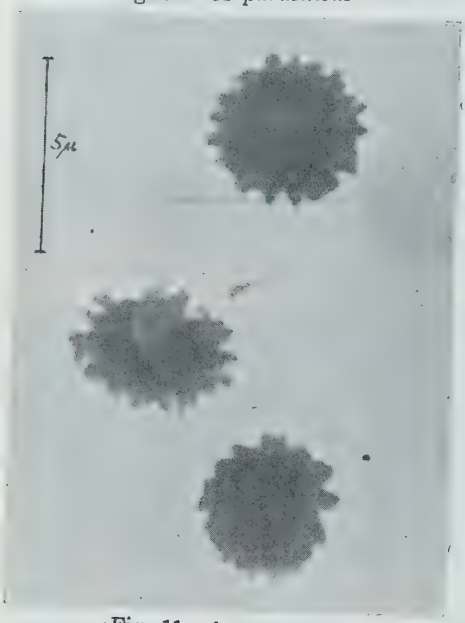
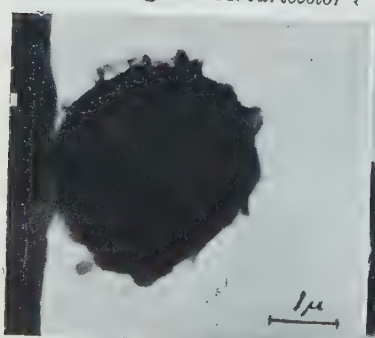


Fig. 11 *A. variecolor*

Fig. 14. *A. itaconicus*



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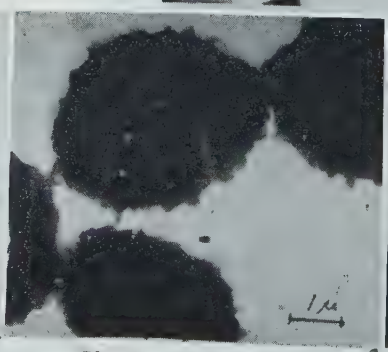

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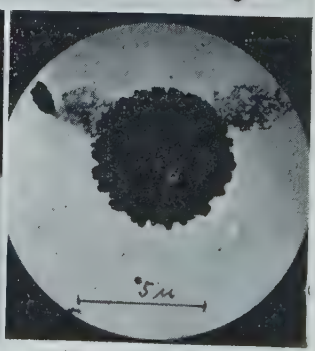
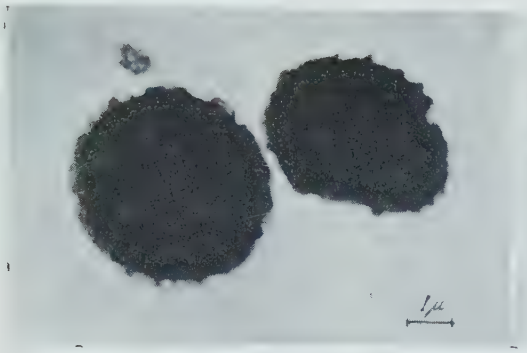


Fig. 17. *A. sojae*



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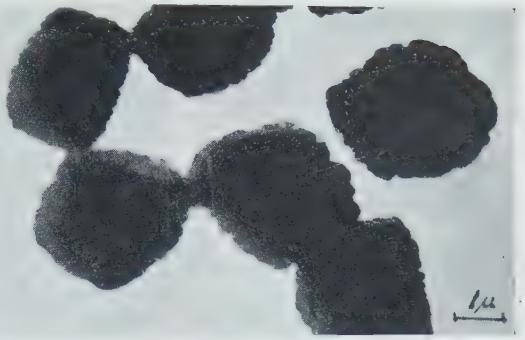

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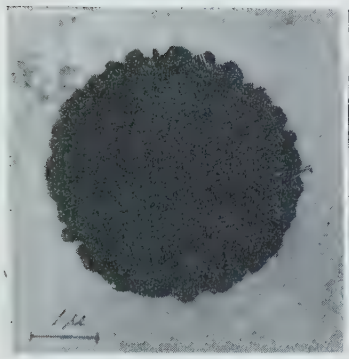

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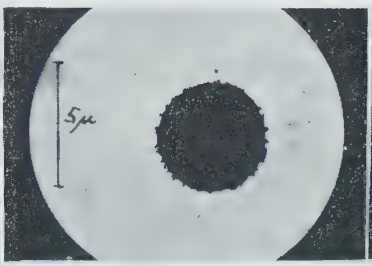

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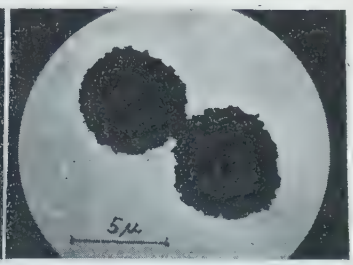

Fig. 21. *A. oryzae*

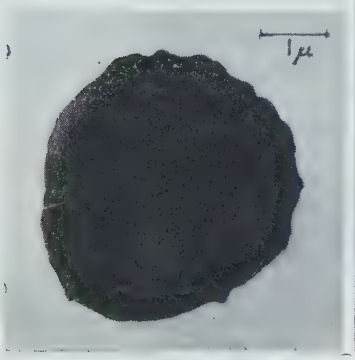

Fig. 23. *A. awamori*

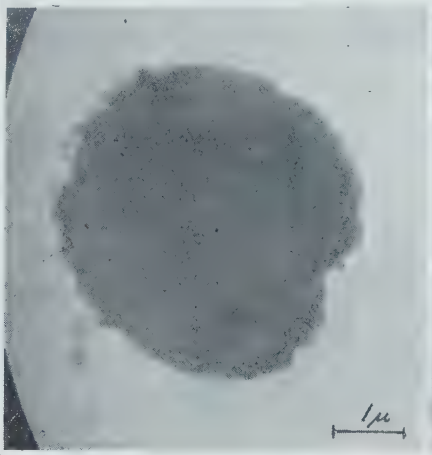

Fig. 24. *A. wentii*

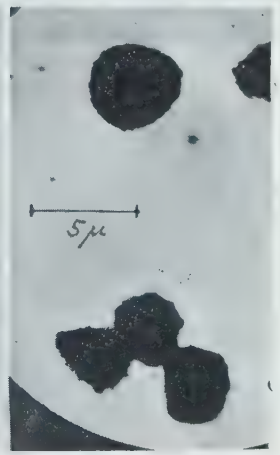

Fig. 25. *A. elegans*

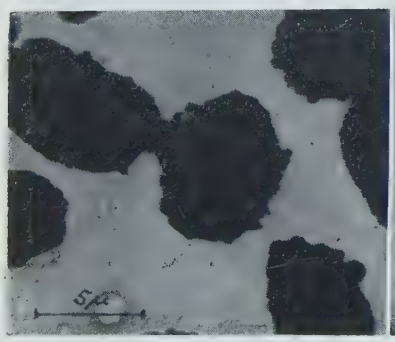

Fig. 26. *A. medius*

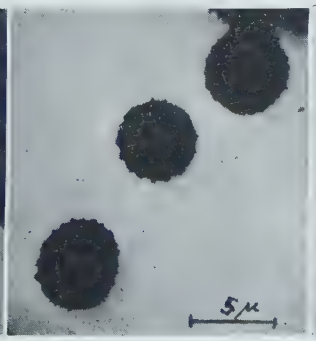

Fig. 27. *A. restrictus*

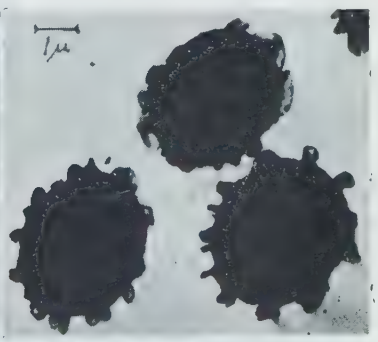

Fig. 28. *A. niger mut. schiemanni*

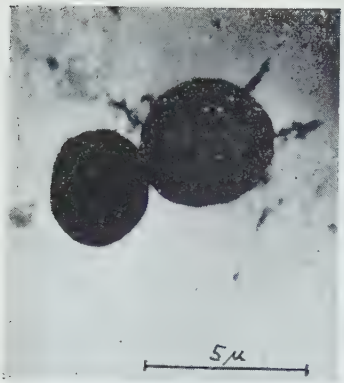

Fig. 29. *A. niger mut. cinnamomeus*

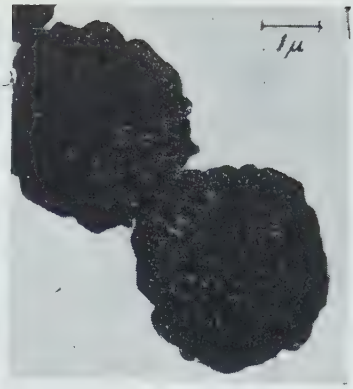

Fig. 30. *A. melleus*

Fig. 31. *A. sydowi*

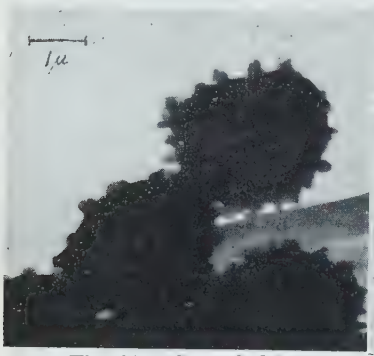

Fig. 32. *A. versicolor*

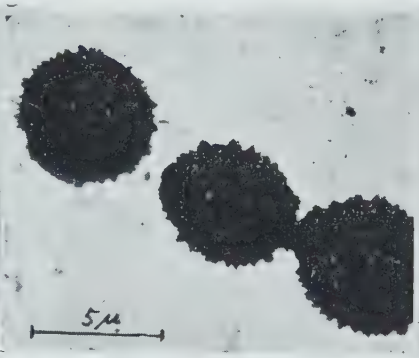

Fig. 33. *A. repens*

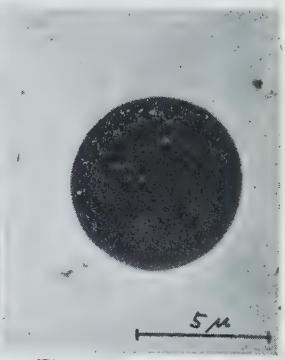

Fig. 34. *A. candidus*

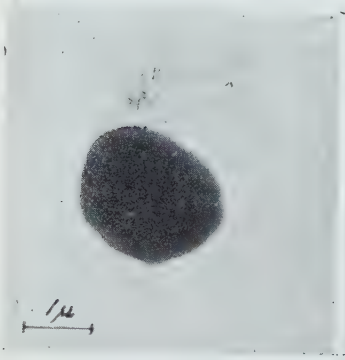

Fig. 35. *A. sulphureus*

Fig. 36. *A. flavipes*

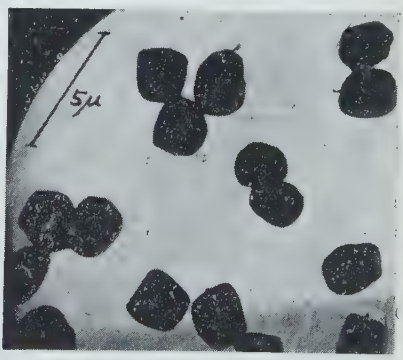

Fig. 37. *A. terreus*

Fig. 38. *A. clavatus*

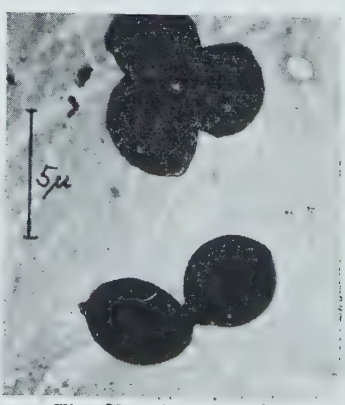

Fig. 39. *A. giganteus*

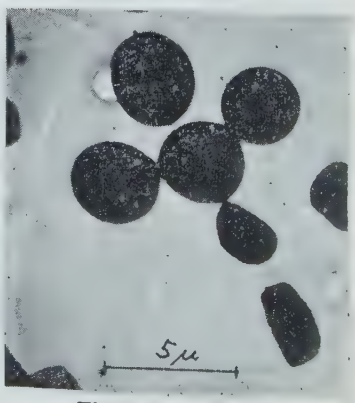

Fig. 40. *A. alliaceus*

SUMMARY

In order to systematize the descriptive terms of the conidial walls, which are one of the essential characteristics in the taxonomy of the Aspergilli, the author classified the micromorphological characteristics of the conidia as observed with an electron microscope.

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STUDIES ON OXIDATIVE FERMENTATION

I. ON THE FORMATION OF NEW SUBSTANCES OF POSITIVE FERRIC CHLORIDE REACTION BY *GLUCONOACETOBACTER LIQUEFACIENS* FROM GLUCOSE

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INTRODUCTION

It is known that formic, acetic, lactic, succinic, glycolic, D-gluconic, 2-keto-D-gluconic, 5-keto-D-gluconic, and 6-oxo-gluconic acids are produced from glucose by *Acetobacter* and *Gluconobacter*.

Recently, Kamlet[5] reported that *Acetobacter suboxydans* produced tartaric acid from glucose via 5-keto-D-gluconic acid when the pH was adjusted to 5.0–7.0 by the addition of alkali.

In 1950, Bernhauer and Riedl-Tumova[1] reported the formation of another as yet unidentified oxogluconic acid besides 5-keto-D-gluconic acid from glucose by *Acetobacter melanogenum*.

Later, Kulka and co-workers[7] (1951) found that *Acetobacter acetosum* formed tartronic acid when it was inoculated for one month at 38° and afterwards allowed to stand for 5 months at room temperature.

As to pseudooxidative bacteria, it is known that D-gluconic-, 2-keto-D-gluconic-, α-ketoglutaric-, pyruvic- and succinic acids are produced from glucose by *Pseudomonas* and that formic-, acetic-, 2-keto-D-gluconic-, α-ketoglutaric-, citric-, L-malic-, lactic- and succinic acids are produced from glucose by *Serratia marcescens*.

The authors have been studying the metabolic products of various sugars produced by the oxidative bacteria, such as *Gluconobacter*, *Pseudomonas* and *Serratia*. In this paper, particular attention was given to *Gluconoacetobacter liquefaciens*, which produced a reddish brown pigment in a culture of glucose solution, and whose sugar metabolism appeared to be peculiar. Among its metabolic products from glucose, tartronic acid and three other substances which gave reddish violet color reactions with ferric chloride were isolated as crystals, and two of the latter were found to be new γ-pyrone derivatives. The formation of γ-pyrone compounds as the metabolic products of microorganisms was almost unknown except for kojic- and comenic acid. The fact that new γ-pyrone compounds were produced by the oxidative bacteria seemed to suggest an important problem

on the mechanism of sugar oxidation in microorganism.

Concerning the formation of substances by *Acetobacter* and *Gluconobacter* which give reddish violet color reactions, Takahashi and Asai[8][9][10] reported the formation of comenic acid by *Gluconobacter liq.* nov. sp. from galactose, and kojic acid by some *Gluconobacter* from mannit, fructose, and sucrose. Kondō *et al.*[6] also found the formation of kojic acid from sorbose. And Ikeda[4] reported the formation of reductone from fructose, glycerine and glycerinaldelhyde by some *Gluconobacter*. But the substances, obtained by the authors are quite different from the compounds described above.

EXPERIMENTAL

1. Oxidation of glucose by Gluconoacetobacter liquefaciens.

Gluconoacetobacter liquefaciens which was isolated by T. Asai was used throughout the experiment. The fermentation was carried out in a medium of 0.8% yeast extract in water containing 9.3% glucose. 100 ml portions of this medium were dispensed in 500 ml shaking flasks. 2.5 g of sterilized $CaCO_3$ was added to each flask just before inoculation. Inoculum was prepared by growing *Gluconoacetobacter liquefaciens* on the koji agar slant containing $CaCO_3$ for 3 days at 30°. Cells of five culture tubes were suspended in 40 ml of sterilized water and 1 ml of the suspensions was inoculated per flask. These cultures were incubated at 30° on a reciprocating shaker at the rate of 150 r.p.m. The pH was determined with "Tōyō" pH test paper, reducing power was shown by ml. of N/10 $Na_2S_2O_3$ solution consumed per ml. of fermentation broth by Lehman-Schoorl method, and Ca-dissolved was determined by ordinary volumetric method. Analytical data is shown in Fig. 1. As seen in Figure 1, the reducing power of Fehling's solution dropped rapidly after 1, 2 and 3 days, and thereafter decreased gradually. As shown by the curve of Ca-dissolved, organic acids were produced rapidly on the 1st and 2nd day,

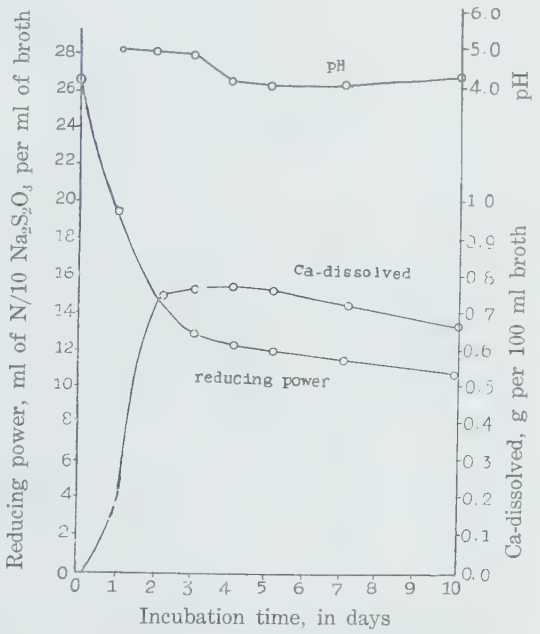
Reducing power, ml of N/10 $Na_2S_2O_3$ per ml of broth
Ca-dissolved, g per 100 ml broth
pH
Ca-dissolved
reducing power
Incubation time, in days
0 1 2 3 4 5 6 7 8 9 10
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
4.0 5.0 6.0

Fig. 1. Oxidation of D-glucose by *Gluconoacetobacter liquefaciens* by shaking culture ($CaCO_3$ added).

reached maximum production on the 4th day and decreased gradually thereafter. Color of fermentation broth was at first slightly yellow, but gradually it turned to reddish brown and finally it became dark brown. Culture liquor changed to reddish violet when some drops of ferric chloride solution were added to it. This was first detected on the 3rd day, became stronger on the 4th and 5th day and there was little change thereafter.

2. *Detection of oxidation products by paper chromatography.*

For the detection of metabolic products in broth, the paper chromatographic technique was adopted. A small amount (3–5 ml) of centrifuged fermentation liquor was freed of cations by adding ion exchange resin (Amberlite IR-120) until the oxalate test for calcium was negative. The liquor was then placed dropwise on a strip of "Tōyō" No. 50 filter paper. The papers were developed by the ascending method with a solvent mixture consisting of 4 volumes of n-butanol, 1.5 volumes of formic acid, and 1 volume of water. After drying overnight at room temperature, it was sprayed with 1% solution of brom phenol blue. Organic acids appeared as yellow spots on a blue background. After the yellow spots were marked with pencil, the filter paper was again sprayed with 1% solution of ferric chloride. Spots of

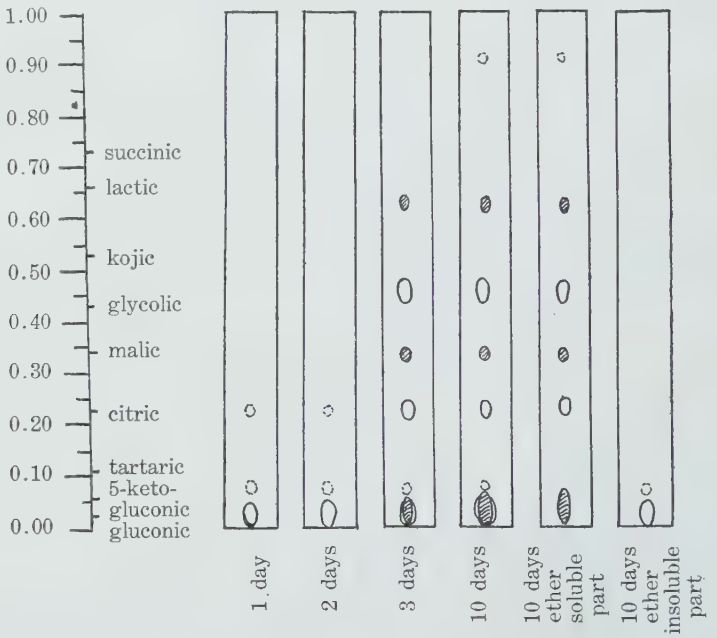

Fig. 2. Paper chromatograms of organic acids produced by *Gluconoacetobacter liquefaciens* from glucose by shaking culture.
Ascending method, solvent (n-butanol 4+formic acid 1.5+H_2O 1)
Reagents: at first BPB 1% sol. in EtOH, and then $FeCl_3$ 1% sol.
Spots: BPB ○ intense, ◌ weak: $FeCl_3$ ●

reddish violet color were detected sharply. The results are shown in Fig. 2. Organic acid, which was produced rapidly on the 1st and 2nd day and dissolved $CaCO_3$ in large quantities, showed an R_F value of 0.02. And the acids which coincide with the R_F values of 0.08 and 0.22 were produced in small quantities. Spots of D-gluconic-, 2-keto-D-gluconic, 5-keto-D-gluconic acid showed R_F values between 0.02 and 0.1, but they could'nt be identified to by R_F values only. On the 3rd day, numbers of spots on paper chromatogram increased. It is especially noticeable that the formation of substances which gave reddish violet color reactions with ferric chloride solution were detected at three positions. The fermentation liquor of the 10th day was extracted with ether and ether soluble and isolube fractions were separately analysed by the above paper chromatographic method. Most of the spots were found in the ether soluble fraction. The R_F values of the culture fluid on the 10th day and its intensity were as follows: S_1 (ether soluble) $=0.02$ (intensity of coloration, ##), S_2 (ether insoluble) $=0.02$ (###), $S_3=0.08$ (+), $S_4=0.22$ (##), $S_5=0.33$–0.35 (+), $S_6=0.47$–0.50 (##), $S_7=0.60$–0.65 (##), $S_8=0.90$ (+). S_4 coincided with glycolic acid. The spots, S_1, S_5 and S_7 were detected by ferric chloride solution.

3. Isolation and identification of metabolic products.

Isolation and identification of metabolic products were conducted with fermentation both after 10 days incubation. Method of separation of the products is shown in Fig. 3. 1.5 L of fermentation broth was centrifuged to separate the residual $CaCO_3$. Then it was boiled and oxalic acid solution was added to precipitate most of Ca ion as Ca-oxalate. The broth was freed of calcium by passing it through the column of cation exchange resin (Amberlite IR-120). The filtrate was concentrated in vacuo and separated to the distillate and the residual part. The distillate was neutralized with lime-water and again concentrated in vacuo, then Ca-salt [Ⅰ] was obtained. For identification of volatile part, another portion (80 ml) of fermentation broth was neutralized with NaOH solution and distilled to obtain the neutral volatile part, then steam distilled after acidifying with H_2SO_4 to obtain and estimate the acidic volatile part.

The residual part (150 ml) was extracted with ether and separated in to ether soluble and insoluble fractions. From the ether soluble part, ether was evaporated and the remainder was separated in to solid and liquid portions.

The solid was dissoved in $NaHCO_3$ solution and extracted with ether, then the extract was recrystallized from water. Crystal [Ⅱ] (mp 203.5°) was obtained. Residual $NaHCO_3$ solution was acidified with H_2SO_4 and extracted with ether. This extract was separated in to crystal [Ⅲ] (mp 230°) and crystal [Ⅳ] (mp 275°) by counter current distribution. The filtrate of the ether soluble part was neutralized with $CaCO_3$ and $Ca(OH)_2$. This was separated in to easily soluble and hardly soluble parts in hot water, i.e. Ca-salt [Ⅴ] and [Ⅵ]. The ether insoluble part was neutralized with $CaCO_3$ and $Ca(OH)_2$, to which methanol (25% conc.) was added in order to precipitate the brown pigment partially, then the solution was fractionally

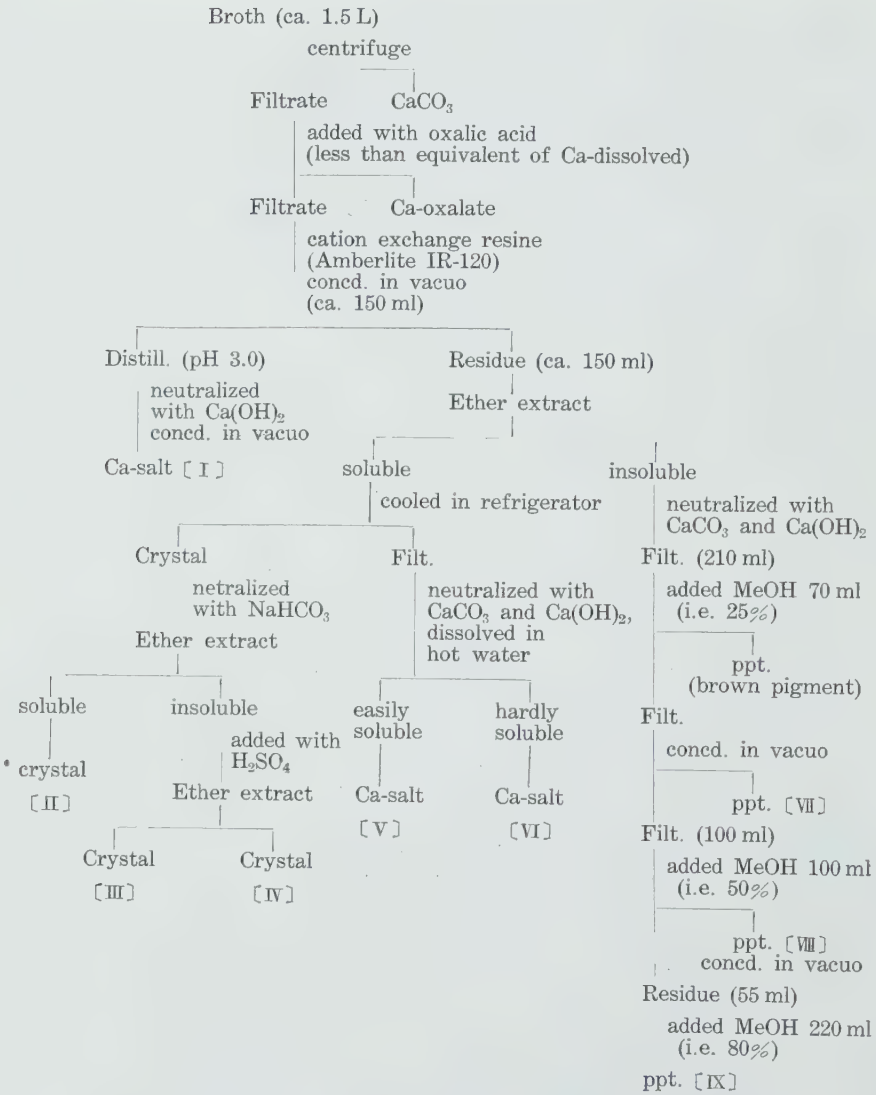

Fig. 3. Separation of organic acids produced by *Gluconoacetobacter liquefaciens* from Glucose.

precipitated by the addition of methanol and separated in to Ca-sat [VII], [VIII] and [IX] by the addition of methanol to various concentrations. Identification of each fractions was conducted as follows:

(A) Volatile part.

(a) Neutral fraction.

(i) Test of ethanol. a) Davy's reaction was negative, b) Agulhon's reaction was negative.

(ii) Detection of aldehyde. a) Tollen's reaction was positive.

(b) Volatile acids.

(i) Detection of formic acid. Sample was neutralized with NaOH, $HgCl_2$ solution added and heated, then a precipitate of white Hg_2Cl_2 was produced. So the existence of formic acid was assumable. Analytical value of formic acid produced was 0.031 g per 100 ml of broth.

(ii) Detection of acetic acid. 2 ml of sample was added with 1 ml of 5% solution of lanthanum nitrate, 1 ml of 0.02 N iodine solution and few drops of ammonia, then its color turned indigo (iodine lanthanum reaction), that suggested the existence of acetic acid. Analytical value of acetic acid produced was 0.012 g per 100 ml of broth.

(B) Ether soluble fraction.

(a) Substances [Ⅱ] (mp 203.5°), [Ⅲ] (mp 230°) and [Ⅳ] (mp 275°) were all isolated as crystals, and gave reddish violet color reactions with ferric chloride. They showed R_F values of 0.60–0.65, 0.2 and 0.47–0.50 respectively. Crystal [Ⅳ] was identified as comenic acid as shown in later report and the other two crystals were found to be new γ-pyrone compounds. Their chemical properties and structures will be reported in the next papers.

(b) Ca-salt, easily soluble in hot water, i.e. Ca-salt [Ⅴ].

(i) The R_F value of this acid was 0.43–0.47 and agreed with that of glycolic acid.

(ii) Elemental analysis of Ca-salt was as follows:

Anal. Calcd. for $Ca(C_2H_3O_3)_2$	C 25.26	H 3.18	Ca 21.08
Found	C 25.15	3.60	20.51

(iii) Free acid was obtained as plate crystal and its melting point was 78°. The melting point was not depressed by admixture with authentic specimen. Therefore this acids was identified as glycolic acid.

(c) Ca-salt, hardly soluble in hot water, i.e. Ca-salt [Ⅵ].

(i) The Ca-salt was hardly soluble both in cold and hot water.

(ii) This was freed from calcium by addition of dilute HCl, placed dropwise on a strip of filter paper, and analysed by the above mentioned method of paper chromatography. The R_F value was 0.22 and near that of citric acid. Sodium nitroprussid reaction and pentabrom acetone reaction of citric acid were tested with this acid but both of these reaction were negative.

(iii) Ca-salt was acidified with H_2SO_4 and extracted with ether. The ether solution was evaporated to dryness and plate crystals were obtained by recrystallisation from ethylacetate. It's decomposition point was 154–156°.

(iv)

Anal. Calcd. for $C_3O_5H_4$	C 30.26	H 2.54	
Found	C 30.21	H 2.90	

(v) 0.0603 g of acid was dissolved in water and titrated with N/10 NaOH solution. Calcd. for $CHOH(COOH)_2$ 10.56 ml. Found, 10.50 ml.

(vii) From above mentioned results this acid seemed to be tartronic acid. Then tratronic acid was synthesized by the method described by

Fenton.[2)(3)] Decomposition point (156°) was not depressed by admixture with authenic specimen of tartronic acid.

With these results, it was identified as tartronic acid.

(C) Ether insoluble fraction.

(a) Precipitate [Ⅵ].

(i) It did not reduce Fehling's solution.

(ii) Orcin and HCl reaction for pentose and 5-keto-D-gluconic acid was negative.

(b) Precipitates [Ⅷ] and [Ⅸ].

These two precipitates were recognized as being the same substances.

(i) Their aqueous solution produced white precipitates by the addition of ammonium oxalate solution, which were not soluble in acetic acid, therefore it is clear that these precipitates are Ca-salts of organic acid.

(ii) The aqueous solution was heated after the addition of orcin and HCl, and it slowly turned bluish green.

(iii) It reduced Fehling's solution and ammoniacal silver nitrate.

4. *Metabolic products from glucose by surface culture of Gluconoacetobacter liquefaciens.*

2 L of 0.8% yeast extract in water containing 6.70% glucose was dispensed into two Fernbach's flasks and sterilized at 120° for 20 min, 25 g of $CaCO_3$ sterilized separately was added to each flask before inoculation.

It was incubated for 40 days at 30°. The color of the fermentation liquor gradually turned to light red, deep red, deep reddish brown, and dark brown. Finally a reddish brown pigment precipitated at the bottom of the flasks. In the course of fermentation, Ca-5-keto-D-gluconate crystallized out. For separation and identification of metabolic products, crystals of Ca-5-keto-D-gluconate were first separated by decantation from the culture liquor, thereafter the fermentation liquor was concentrated in vacuo, cooled in a refrigerator and then Ca-5-keto-D-gluconate was crystallized out again and the mixture was filtered. The filtrate was then treated by methods similar to those described in Fig. 3. After recrystallization, identification of Ca-5-keto-D-gluconate was conducted.

(i) It reduced Fehling's solution.

(ii) Ca-salt was dissolved in conc. HCl and added with orcin, then the color turned to green. This pigment was extracted with amyl alcohol.

(iii) It was analysed for calcium content.

Anal. Calcd. for $C_{12}H_{18}O_{14}Ca2^1/_2H_2O$ Ca 8.6%

Found Ca 8.4%

(iv) The p-nitrophenylhydrazone prepared from this purified calcium salt showed mp 190° and agreed with the literature data. Thus it was demonstrated to be identical with Ca-5-keto-D-gluconate. The fermentation liquor separated from Ca-5-keto-D-gluconate was acidified with H_2SO_4 and extracted with ether. Ferric chloride reaction test was made on the ether soluble part, but it was negative. By paper chromatography

of the fermentation broth, three spots were detected and agreed with the R_F values of the unknown reducing acid, tartronic and glycolic acids. By methods similar to those described in Fig. 3, 4.5 g of Ca-glycolate and 2 g of Ca-tartronate from 1 L of broth were obtained and identified.

5. Oxidation of glucose by shaking culture of Gluconoacetobacter liquefaciens without $CaCO_3$.

0.8% yeast extract in water containing 8.7% glucose was inoculated with *Gluconoacetobacter liquefaciens* and incubated at 27° for 10 days, by shaking culture without $CaCO_3$. Chemical changes during the fermentation process is shown in Fig. 4. After 24 hours, the pH of the fermentation liquor dropped to 2.8 and thereafter it continued to decrease little by little. Acidity was estimated by titration with 1/10 N NaOH solution, which increased slowly day by day. But as shown in the curve of reducing power, the fermentation was very slow. The color of the fermentation liquor remained light yellow and no pigmentation was observed. Only one spot with the R_F value of 0.02 was recognized by paper chromatography, and other acids were not produced. Perhaps the fermentation was inhibited by the low pH, which was caused by the production of organic acids.

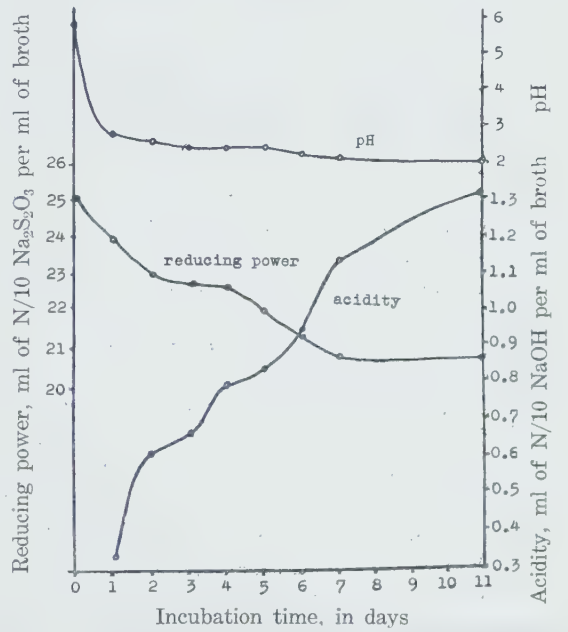
pH
reducing power
acidity
Reducing power, ml of N/10 $Na_2S_2O_3$ per ml of broth
Acidity, ml of N/10 NaOH per ml of broth
pH
Incubation time, in days

Fig. 4. Oxidation of D-glucose by *Gluconoacetobacter liquefaciens* by shaking culture ($CaCO_3$ not added).

6. Oxidation of various sugars by Gluconoacetobacter liquefaciens.

Oxidative products from various sugars by *Gluconoacetobacter liquefaciens* were studied both by surface and shaking cultures. D-fructose, D-mannose, D-galactose, D-xylose, and L-arabinose were dissolved up to 5% in 0.8% yeast extract in water, and 100 ml per flask was dispensed into 500 ml shaking flasks. 1.25 g of sterilized $CaCO_3$ was added to each flask just before inoculation. These cultures were incubated at 30° for 49 days by surface culture and at 27° for 10 days by shaking culture. Analytical data of surface culture are shown in Table 1.

Table 1. Oxidation of various sugars by surface culture of *Gluconoacetobacter liquefaciens*.

No.	Initial sugar				Fermented liquor**		
	Substrate	Content %	Reducing power*	pH	Reducing power	Ca-dissoved (g/100 ml)	Color of broth
1	D-fructose	12.76	4.5	6.4	8.5	0.16	brown
2	D-mannose	13.45	4.5	6.6	3.4	0.75	brown
3	D-galactose	12.54	4.5	6.2	1.2	0.18	brown
4	D-xylose	14.91	5.0	6.0	6.7	0.80	brown
5	L-arabinose	13.33	4.3	6,6	0.8	0.90	brown

* Reducing power: ml of N/10 $Na_2S_2O_3$ solution comsumed per ml of broth by Lehman Schoorl's method.

** Cultures were harvested at the 49th day.

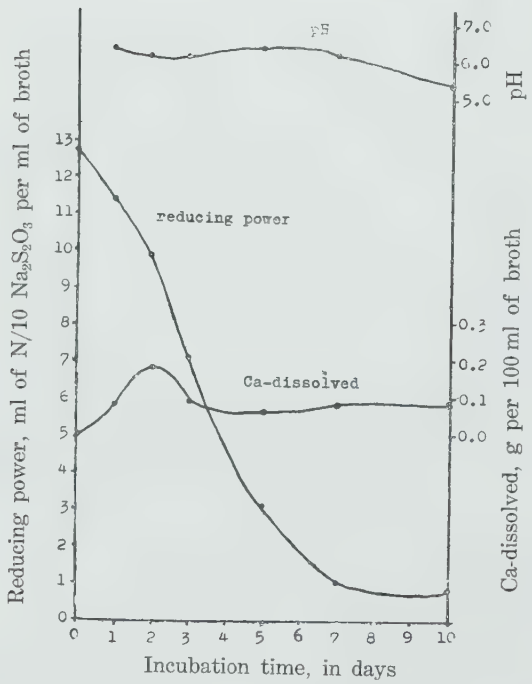
pH
reducing power
Ca-dissolved
Reducing power, ml of N/10 $Na_2S_2O_3$ per ml of broth
Ca-dissolved, g per 100 ml of broth
Incubation time, in days

Fig. 5. Oxidation of D-fructose by *Gluconoacetobacter liquefaciens* by shaking culture.

Analytical data of shaking culture are shown in Fig. 5, 6, 7 and 8.

Detection of organic acids produced from these sugars was tried by paper chromatography.

D-fructose: Production of organic acid was very small and only one spot was found on the paper chromatogram. Its R_F value was 0.02.

D-mannose: Reducing power decreased rapidly and increased again. Only one spot was found on the paper chromatogram and agreed with that of mannonic acid.

D-galactose: Reducing power decreased rapidly. Only one spot was found on the paper chromatogram. Its R_F value was 0.08 and agreed with that of galactonic acid.

D-xylose: Reducing power decreased rapidly and increased again. Three spots were found on the paper chromatogram, corresponding to R_F

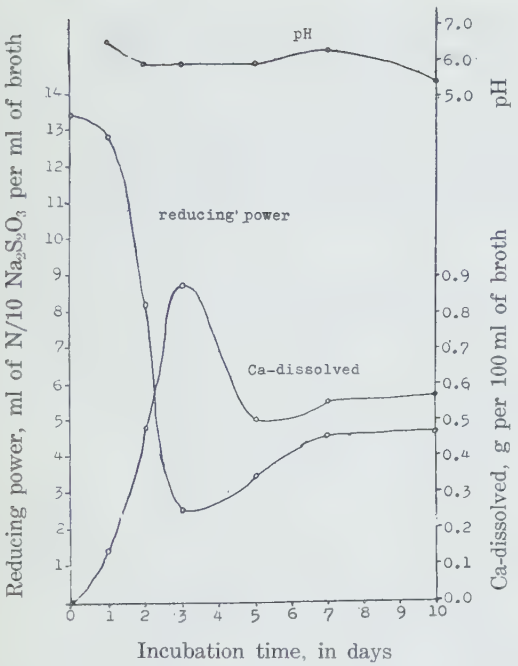

Fig. 6. Oxidation of D-mannose by *Gluconoacetobacter liquefaciens* by shaking culture.

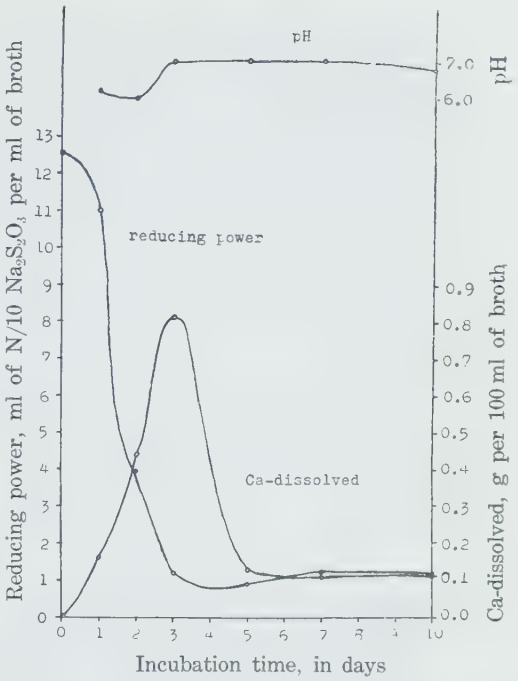

Fig. 7. Oxidation of D-galactose by *Gluconoacetobacter liquefaciens* by shaking culture.

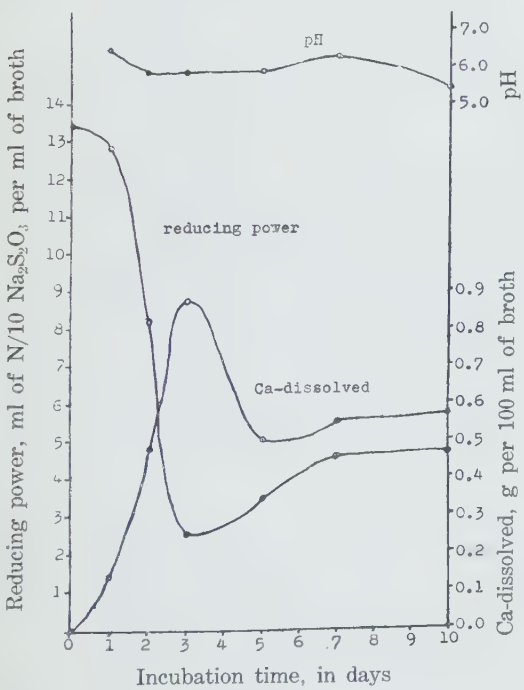

Fig. 8. Oxidation of D-xylose by *Gluconoacetobacter liquefaciens* by shaking culture.

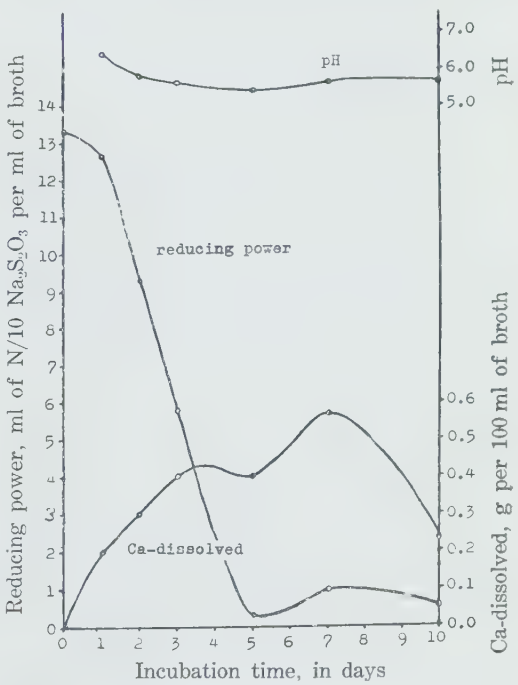

Fig. 9. Oxidation of L-arabinose by *Gluconoacetobacter liquefaciens* by shaking culture.

values of 0.08, 0.28, and 0.37. The spot which showed R_F value of 0.08 agreed with that of xylonic acid. Other two substances are now under investigation.

L-arabinose: Two spots with the R_F values of 0.06 and 0.23 were found on the paper chromatogram. Ca salt of organic acid crystallized out in the quantity of 2 g per 100 ml of broth when it was allowed to stand at room temperature. The aqeous solution of this crystal was heated to 150–160° after addition of conc. H_2SO_4 and isatin, then it turned to a light reddish violet color. The R_F value of this acid was 0.06 and agreed with that of L-arabonic acid. From the data it was presumed to be L-arabonic acid.

From the above mentioned sugars, we could not find the formation of the substances which gave the reddish violet color reaction with ferric chloride, and none of the γ-pyrone compounds which we obtained from glucose from this organism were detected by paper chromatography.

SUMMARY

(1) The authors studied the oxidation of D-glucose, D-fructose, D-mannose, D-galactose, D-xylose, and L-arabinose by surface and shaking cultures of *Gluconoacetobacter liquefaciens*.

(2) The production of organic acids from these sugars was studied by paper chromatographic techniques and 8 spots from D-glucose, one spot from D-fructose, D-mannose, D-galactose, 3 spots from D-xylose, 2 spots from L-arabinose were detected on the paper chromatograms. The production of D-mannonic acid from D-mannose, D-galactonic acid from D-galactose, D-xylonic acid from D-xylose, and L-arabonic acid from L-arabinose were assumed.

(3) The oxidation of glucose was studied in detail. When $CaCO_3$ was not added to the shaking culture, glucose was oxidized very slowly. When $CaCO_3$ was added to the shaking culture, glucose was oxidized rapidly and the organic acid with the R_F value of 0.02 was produced at first and then the production of organic acids with the R_F values of 0.22–0.25, 0.33–0.35, 0.43–0.47, 0.60–0.65, and 0.90 followed. Three spots with the R_F values of 0.02, 0.47–0.50, and 0.60–0.65 were detected with ferric chloride solution.

(4) Isolation and identification of oxidative products from glucose were tried. Aldehyde, formic-, acetic-, 5-keto-D-gluconic-, glycolic-, and tartronic acid, other unknown reducing acids and three substances which gave reddish violet color reactions were isolated from the culture liquid by shaking culture.

(5) Three substances of positive ferric chloride reaction were isolated as crystals, showing the melting point of 203.5°, 230° and 275°, respectively. All of them were recognized as γ-pyrone compounds and the former two were recognized as new substances. Since these γ-pyrone compounds were found to be produced by other oxidative bacteria, for example by *Aceto-*

bacter melanogenum, which produced reddish brown pigment in the fermentation liquor, these new substances were named "Rubiginol" and "Rubiginic acid" ("rubiginosus" means "reddish brown").

(6) These three γ-pyrone compounds were found to be produced from D-glucose by *Gluconoacetobacter liquefaciens* when it was incubated by shaking culture with the addition of $CaCO_3$, but not without $CaCO_3$. They were not produced from glucose by surface culture both with and without $CaCO_3$.

From D-fructose, D-mannose, D-galactose, D-xylose, and L-arabinose, we could not find the formation of the substances of positive ferric chloride reaction both in shaking and in surface culture.

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STUDIES ON OXIDATIVE FERMENTATION
II. ON THE NEW γ-PYRONE COMPOUND, RUBIGINOL, PRODUCED BY *GLUCONO-ACETOBACTER-LIQUEFACIENS*

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INTRODUCTION

The formation of γ-pyrone compounds as the metabolic products of micro-organisms seldom occurs except for kojic acid, which was discovered by a Japanese investigator K. Saito[3] in 1907, the constitution of which was decided by T. Yabuta[9][10][11] in 1924. It is well known that the Aspergillus oryzae-flavus-tamarii group produces a large amount of kojic acid from sugars. On the other hand, in 1933 Takahashi and Asai[4][5][6] confirmed that kojic acid was also produced by the oxidative bacteria from nannitol and fructose but not from glucose. Kondō[2] recently reported its formation from sorboe by the *Gluconobacter* species. Another γ-pyrone compound, comenic acid was found and identified by Takahashi and Asai[7][8] in the fermentation products of galactose by *Gluconobacter liquefaciens*.

The author, in the course of a study on the metabolic products of *Gluconobacter*, *Pseudomonas*, and *Serratia*, found that three γ-pyrone compounds were produced in the culture liquid of *Gluconoacetobacter liq.* with a medium of 0.8% yeast extract in water containing 9.3% glucose and 2.5% $CaCO_3$ by shaking culture but not by surface culture. Two of these compound were confirmed to be new γ-pyrone compounds, to which the names of "Rubiginol" and "Rubiginic acid" were given.

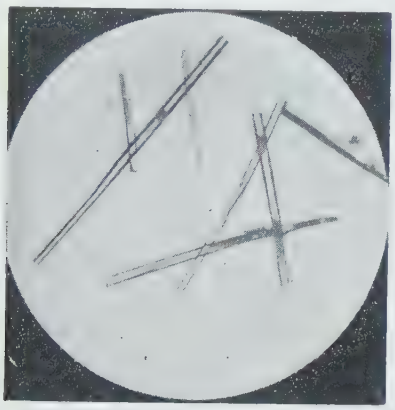
The present paper reports the experimental results dealing with the chemical properties of rubiginol and its chemical structure which has been decided as 3,5-dihydroxy-1,4-pyrone.

EXPERIMENTAL

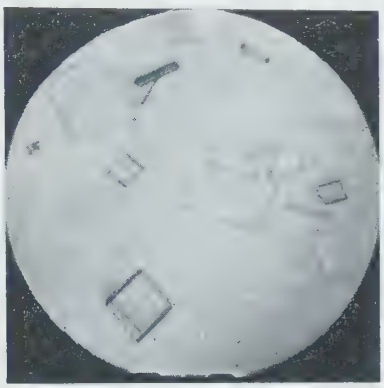
1. *Properties of rubiginol.*

Rubiginol was obtained as colorless plate crystals. When its aqueous solution was cooled rapidly, it crystallized out as prismatic needles which contained one molecule of water of crystallization, but the water of crystallization was lost easily at room temperature. It melts at 203.5° (uncorr.). It is easily soluble in pyridine, dioxane, acetone, methanol and ethanol, soluble in water, isopropanol, butanol, galcial acetic acid, ethylacetate and

ether ; and scarcely soluble in toluol, xylol and benzol. Its aqueous solution is slightly acidic and reduces Fehling's solution and ammoniacal silver nitrate solution. Light green copper salt precipitates out from the aqueous solution upon the addition of copper acetate solution. It decolorizes bromine water and potassium permanganate solution. From the above mentioned facts, it seemed to be a weak acid belonging to phenolic or enolic compounds which contains at least one carbonyl group and at leat one double bond.



(a) Rubiginol (+1 H_2O) ×400



(b) Rubiginol dimethyl ether ×400

Fig. 1. Photographs of crystals.

2. *Elemental analysis.*

After it was dried by Abderhalden's apparatus, elemental analysis was conducted. It was freed from nitrogen and sulphur.

Analysis.	Found	C	46.92	H	2.96
			46.90		3.29
	Calcd. for $C_5H_4O_4$	C	46.88	H	3.15

Accordingly, the empirical formula was given as $(C_5H_4O_4)_n$.

3. *Determination of molecular weight.*

Molecular weight was determined by Rast's method. Found 144 Calcd. for $C_5H_4O_4$ 128.

Molecular weight was culculated as 128 from potentiometric titration curve. Molecular formula was then decided as $C_5H_4O_4$.

4. *Infra-red absorption spectrum of rubiginol.*

Infra-red recording spectrophotometer, Baired model B No. 143 was used. The absorption spectrum of infra-red ray showed the existence of a secondary or phenolic OH group, possibly a conjugated carbonyl group in its structure, while the existence of primary or tertiary OH groups, lactone or carboxyl groups were negated.

5. *Ultraviolet absorption spectrum.*

Ultraviolet absorption spectra of diacetylrubiginol and diacetyl kojic

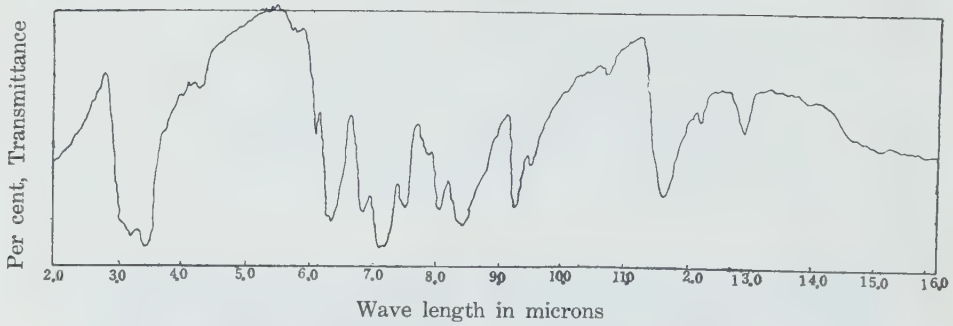
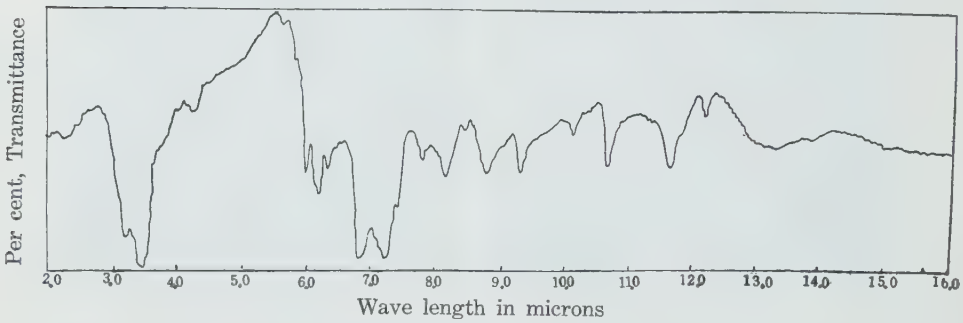


Fig. 2. Infrared absorption spectrum of rubiginol.



Fg. 3. Infrared absorption spectrum of kojic acid.

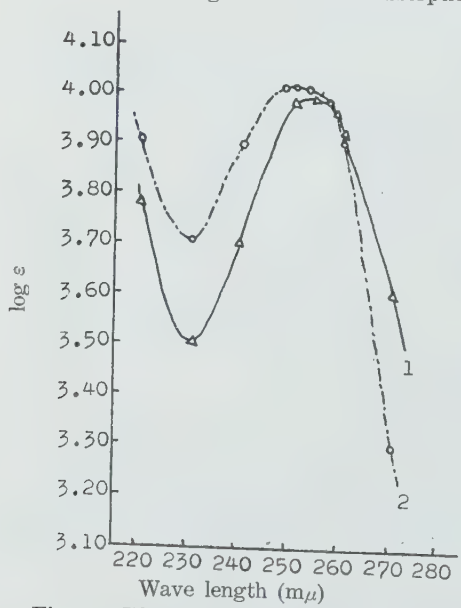


Fig. 4. Ultraviolet absorption spectra
1 : diacetylkojic acid 2 : diacetylrubiginol

acid were compared. As shown in Fig. 4, both spectra were very similar. Maximum absorption was observed at 250 mμ with diacetyl kojic acid and at 255 mμ with diacetylrubiginol. Sin e the maximum absorption of furan derivatives generally occures at longer wave lengths, it was concluded that rubiginol is more similar to kojic acid than to furan derivatives.

6. *Function of oxygen atoms.*

The substance contains four oxygen atoms in its structure, and it is certain that at least one of them exists as a carbonyl group.

To ascertain the behavior of the other oxygen atoms, the existence and the number of OH groups were examined.

(*a*) *Methylation.* 1 g of sample was dissolved in 8.4 ml of 10% NaOH solution and was shaken after the addition of 2 ml of dimethylsulfate. After a while the methylated compound crystallized out. This was recrystallized from methanol, and colorless plate crystals which melted at 143° were obtained, yield, 730 mg and it showed no color reaction with ferric chloride. It does not show an acidic reaction.

Analysis.	Found	C	53.91	H	4.85
			53.81		4.77
	Calcd. for $C_5H_2O_2(OCH_3)_2$	C	53.86	H	5.16
Analysis.	Found	OCH_3	39.61		
	Calcd. for $C_5H_2O_2(OCH_3)_2$		39.74		

(*b*) *Acetylation.* 100 mg of rubiginol was dissolved in 0.5 ml of acetylanhydrid and heated at 100° under reflux cooler for 2 hours. After the reaction ceased, acetylanhyrid was decomposed by the addition of water, the acetic acid was neutralized with NaOH solution, and the acetylated compound was extracted with ether. After removal of the solvent, the extract was recrystallized from methanol and colorless crystals were obtained, mp 118°. However, after it was heated with NaOH solution and acidified with HCl it again showed reddish violet color reaction with ferric chloride.

Analysis.	Found	C	51.39	H	4.16
			51.33		3.85
	Calcd. for $C_5H_2O_2(OCOCH_3)_2$	C	50.95	H	3.80

It agreed with diacetyl rubiginol.

From the results mentioned above, it was concluded that rubiginol has two hydroxyl groups in its structure which represent the acidic and ferric chlorid reactions.

(*c*) *Potentiometric titration of rubiginol.*

Potentiometric tiration curves of rubiginol and kojic acid are shown in Fig. 5. Rubiginol acted as a dibasic acid, pK value of the stronger acid group was 7.62 which was almost the same as that of kojic acid, 7.94 and the pK value of weaker acid group was 10.48. The substance was easily extracted from its $NaHCO_3$ solution with ether and could be set free by carbon dioxide. Therefore it is clear that the substance does not have a carboxyl group in its chemical structure and moreover the acidity and ferric chloride reaction are due to the existence of these two hydroxyl groups.

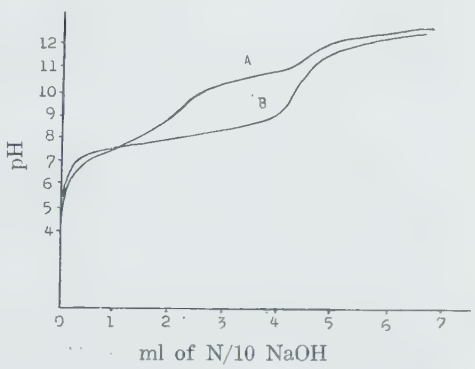
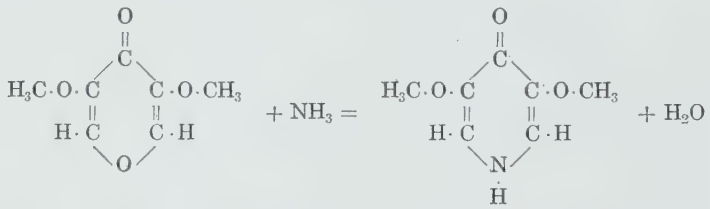


Fig. 5. Potentiometric titration curves of rubiginol and kojic acid
A : Rubiginol 59.4 mg in 100 ml H_2O
B : Kojic accid 61.7 mg in 10 ml H_2O

7. *Existence of γ-pyrone ring.*

Twenty seven chemical structures could be given for rubiginol from its molecular formula as ring compounds, i.e. α-pyrone, γ-pyrone, and furan derivatives. But in many respects rubiginol was found not to be an α-pyrone or furan derivative. As mentioned above, rubiginol is very similar to kojic acid with respect to its properties, the author assumed that it belongs to γ-pyrone compound. The proof of the existence of γ-pyrone ring in its chemical structure was conducted by transforming it into a pyridone derivative.



Diluted ammonia water was added to the dimethyl compound and heated on a water bath, the reaction product was obtained as colorless prismatic crystals when it was recrystallized from water. It melted at 270° and the results of its elemental analysis coincided with that of the corresponding pyridone derivative.

Analysis.	Found	C	53.68	H	5.87	N 9.24
	Calcd. for $C_7H_9O_3N$	C	54.19	H	5.85	N 9.03

Infra-red absorption spectrum of this compound also showed the existence of NH-group (Fig. 6).

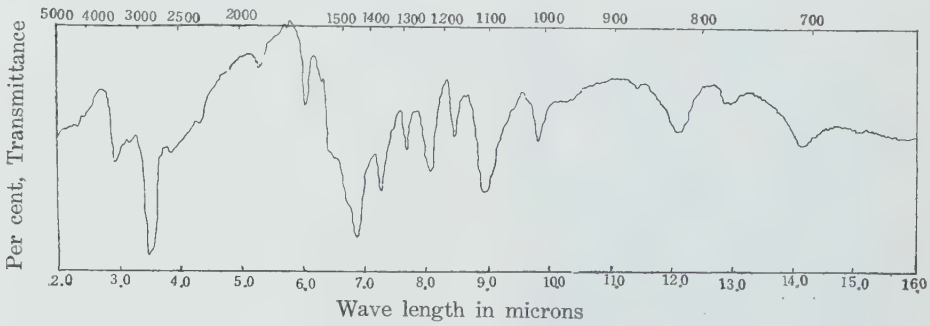


Fig. 6. Infrared absorption spectrum of pyridone drivative produced from dimethylrubiginol.

8. *Position of hydroxyl groups.*

If rubiginol is one of the γ-pyrone derivatives, four different chemical structures should be given to it according to its molecular formula as shown in Fig. 7.

Among these, the fourth substance, viz. 2,4-dihydroxy-1,4-pyrone had been already reported by R. Kaushel[1] in 1940 and is clearly different from my substance according to its melting point (94°). In accordance

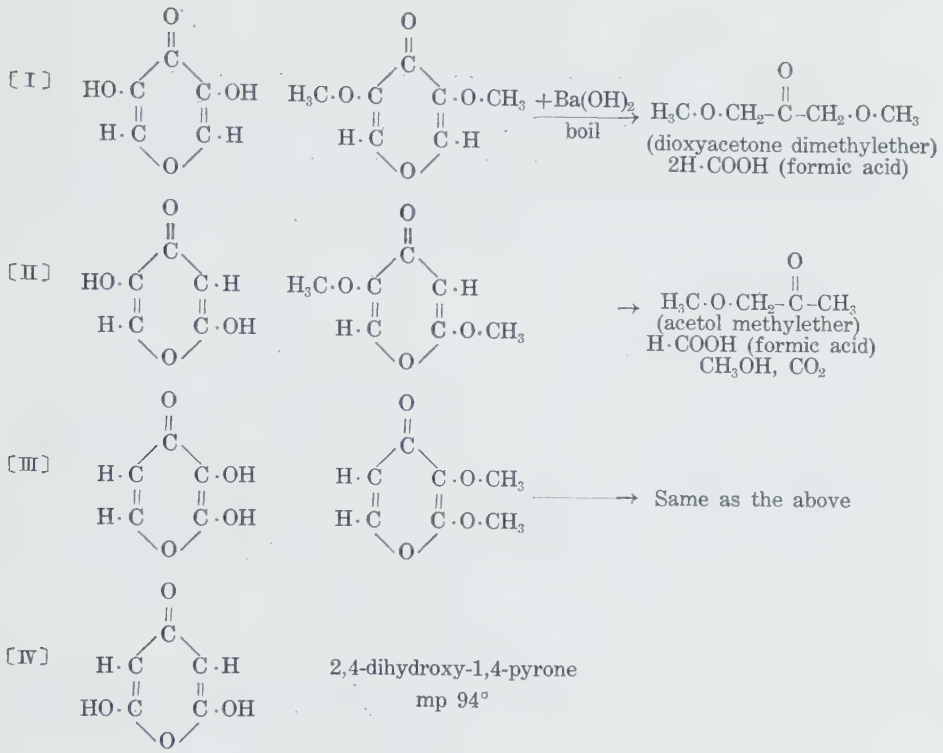


Fig. 7. γ-Pyrone derivatives having molecular formula $C_5H_4O_4$ and the decomposition products of their methylethers.

with the method adopted by provious investigators to decide the chemical structures of γ-pyrone derivatives, its dimethyl ether was hydrolized with barytha water in the hot state and then the decomposed products were subjected to analysis. The distilled fraction of the decomposition products of the second and third substances in Fig. 7, must be the same as that of kojic acid, i.e. acetolmethylether. The decomposition products of dimethyl-kojic acid was distilled and acetolmethylether was identified as its p-nitro-phenylhydrazone, mp 111°, which coincided with Yabuta's investigation and 2,4-dinitrophenylhydrazone was also pepared which melted at 162°. On the other hand, the distilled fraction of decomposition products of dimethyl-rubiginol gave a different 2,4-dinitrophenylhydrazone, which melted at 121°, suggesting that only the first chemical structure might be possible for the substance. In fact, from the elemental analysis of this 2,4-dinitrophenyl-hydrazone, the carbon, hydrogen, nitrogen, and methoxyl contents agreed perfectly with those of dioxyacetone dimethylether.

Analysis.	Found	C 44.17	H 4.40	N 19.26
		44.32	4.38	19.05
	Calcd. for $C_{11}H_{14}O_6N_4$	C 44.27	H 4.73	N 18.79

Found		OCH_3	20.76
			20.68
Calcd. for $C_9H_8O_4N_4(OCH_3)_2$		OCH_3	20.81

Residue of distillate was acidified with sulphuric acid and distilled in steam. The distillate was acidic and produced a white precipitate upon the addition of $HgCl_2$ solution in the hot state. The distillate was neutralized with $CaCO_3$ and $Ca(OH)_2$ and concentrated *in vacuo*. Ca salt was obtained by recrystallization as colorless plate crystals.

Analysis.	Found	Ca	30.61
			30.78
	Calcd. for $(HCOO)_2Ca$	Ca	30.80

It agreed with Ca formate. From 400 mg of rubiginoldimethylether, 20.6 mg of formic acid was found to be produced by titration and 21.4 mg of Ca formate was obtained, so it was concluded that 1 mol. of of dioxyacetonedimethylether and 2 mol. of formic acid were produced from 1 mol. of dimethylrubiginol.

From the experimental results mentioned above, rubiginol was confirmed to having the structure formula [1] in Fig. 7, i.e. 3,5-dihydroxy-1,4-pyrone.

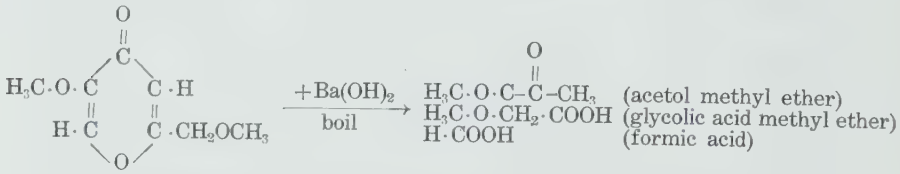


Fig. 8. Decomposition products of dimethylkojic acid.

SUMMARY

In the previous report, the authors found the formation of three substances which gave reddish violet color reactions with ferric chloride in the metabolic products of glucose by *Gluconoacetobacter liquefaciens*. One of them was found to be a new γ-pyrone derivative and was named as rubiginol. Its chemical properties was studied and the chemical structure was confirmed to be 3,5-dihydroxy-1,4-pyrone.

ACKNOWLEDGEMENTS

I wish to express my hearty thanks to Prof. T. Asai for his kind guidance and encouragement in this work, and am also indebted to Dr. T. Yabuta, Prof. Y. Sumiki, Prof. K. Sakaguchi, Director of the Institute of Applied Microbiology and Dr. Y. Ikeda for their kind suggestions and Mr. H. Kamata for his help with infrared absorption spectra., and to the microanalysers of the Dep. of Agr. Chem., Fac. of Agr., Univ. of Tōkyo.

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PECTIC GLYCOSIDASES OF *ASPERGILLUS NIGER*

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The theory that various hydrolytic enzymes are involved in the glycoside breakdown of polygalacturonic acid was first developed many years ago. In the course of pectic glycosidase formation by *Aspergillus niger*, there seemed to be some inconsistency between the development of activities determined by viscosity-reduction and by reducing-group-liberation, so that two different types of glycosidases were thought to be produced by this mold[1]. One of them rapidly reducing the viscosity of the substrate, pectic acid, rapidly compared to the reducing group-liberation, is provisionally called depolymeric polygalacturonase (DPG), and the other, appearing to act *vice versa*, is called galacturonogenic polygalacturonase (GPG). Further information about these two enzymes[20], production, partial purification, and properties, is summarized on the present report.

METHODS

Spores of a strain of *Aspergillus niger* (S_3) which had been isolated from ramie fiber[1] were taken from koji (rice bran extract) agar slant, inoculated into Czapek's synthetic media containing 3% of C-source and shaken reciprocally at 30°. Glucose for injection use or citrus pectin which contains 65% uronic anhydride and 7.8% methoxyl were used for C-sources.

Determinations of viscosity-reducing activity were carried out in Ostwald's viscosimeters. The enzyme action was expressed as percent viscosity change at 30°, which was calculated as follows[24]:

$$A_{n\ \mathrm{hr}} = \frac{T_0 - T}{T_0 - T_a} \times 100$$

T_0=flow time in seconds of 1% pectic acid solution (4 cc)+inactivated enzyme (1 cc), or initial flow time extrapolated from the curve of viscosity-reduction by the enzyme action. T_a=flow time in seconds of pure water+if necessary, inactivated enzyme. T=flow time in seconds at reaction time, n hours, of pectic acid solution+active enzyme.

The pectic acid used in the determination was prepared by NaOH-deesterification of citrus pectin and twice precipitated with ethanol. The pectic acid contains 69% uronic anhydride. This was dissolved in 1% solution buffered at pH 4.2 with half a volume of McIlvaine's buffer.

When the culture filtrate was used as the enzyme solution, the ratio of dilution to get a result of a suitable A value was shown in parentheses. Since this value A was not proportional to the quantity of enzyme, if quantitative comparison was necessary, enzyme activities were expressed in DPGu determined from Fig. 1, which is based on units similar to

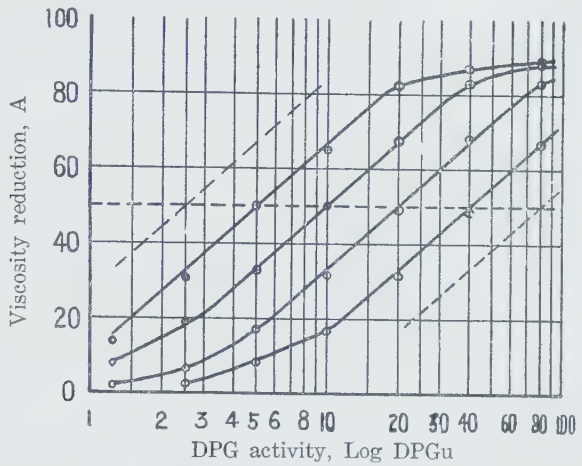


Fig. 1. Standard curves of relation between A value and DPGu at various reaction times

Curves mean DPGu in response to A value of following reaction times: from the left, 4 hours (dotted), 2 hours, 1 hour, 30 minutes, 15 minutes and 7.5 minutes (dotted), respectively. Dotted lines are assumed.

Schubert's (GEv) unit[21]. In the determination procedure, the enzyme solution was diluted or the reaction time was elongated appropriately in order to obtain an A value between 20~80%, the linear range.

The activity of reducing group-liberation was determined by the iodometric method of Jansen and MacDonnell[6], but since the apparent activities of the present preparations were far less than in their case, it was expressed, unless otherwise stated, in the milliequivalents of galacturonic acid liberated by 1 cc of the enzyme solution from 20 cc of 1% pectic acid solution, pH 4.2, per given reaction time at 30° (Gal.meq n hr).

RESULTS

Induced and non-induced (constitutive) formation of glycosidases. — As previously reported[1], the development of activities of viscosity-reduction or of reducing group-liberation acting on pectic acid shows the different types of curves according to the C-source, pectin or glucose, fed to the mold culture (Fig. 2). The activity to liberate the reducing groups, mainly due to GPG, was induced by pectic homologous substrate, whereas DPG with regard to the reduction of the viscosity seemed to have no interaction with the C-source and was produced abundantly even with heterologus C-sources at least in the late phase of the culture. The apparent induced

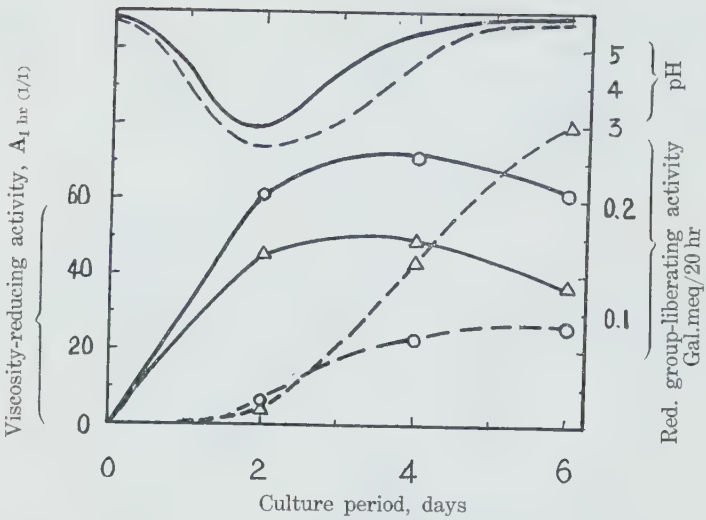


Fig. 2. Figures of pectic glycosidases formation in culture liquor with or without adaptive substrate

Asp. niger was shaken at 30° in Czapek-peptone medium with pectin or pectic acid (solid lines), or with glucose or starch (dotted lines); The change of pH is shown on the upper side; Triangles mean development of viscosity-reducing-activities, and circles, reducing group-liberating activities.

formation of viscosity-reducing activity observed in the early stage of the culture with pectic substrate, was due to the formation of GPG, although in previous reports it remained questionable. GPG is induced even by shaking washed cells with only pectic materials, i.e. without growth, as seen in the predominant appearance of reducing group-liberating activity as compared with viscosity-reducing activity (Table 1, upper columns). This tendency is strikingly revealed by shaking with salt-free water: because of the lower solubility of DPG, its viscosity-reduction activity cannot be observed, but only GPG, which is more soluble in water, is seen to be induced by means of viscosity measurement while shaking the mold cells with the salt-free water containing pectin for a short time (Table 1, lower columns). These properties with regard to the induced formation were confirmed in experiments wherein a series of cultures were used each of which contained different quantities of pectin (Fig. 3). In order to avoid the inactivation or other complicated influences occurring secondarily, the pH value of the culture medium during cultivation was kept at 3~5. The results show that the formation of GPG corresponds to the existent inducer quantitatively, while DPG seems to be a constitutive enzyme, the formation of which depends on other factors. If the viscosity-curves were corrected to include only the DPG activity, then it can be seen that the amount of DPG produced in each serie is inversely related to the amount of the inducer.

Table 1. Effect of substrate and medium on the formation of the enzymes

Medium for shaking	Enzyme activity of the filtrate, shaking time: 5 hours	
	Gal_{meq}/20 hours	$A_{1\ hr\ (1/1)}$
Sörensen's buffer (pH 4.0)	—	26.3
〃 〃 +glucose 0.5%	0.05	38.4
〃 〃 +pectin 0.5%	0.21	44.4
〃 〃 +(glucose 0.25% pectin 0.25%)	0.15	43.4
(DPG purified)	0.06	44.8

Medium for shaking (adjusted to pH 4.0 with HCl)	Enzyme activity of the filtrate, shaking time	
	18 hours	44 hours
	$A_{1\ hr\ (1/1)}$	$A_{1\ hr\ (1/1)}$
Distilled water	2.5	65.3
〃 〃 +glucose 0.5%	3.4	77.6
〃 〃 +pectin 0.5%	21.2	84.8

The washed cells of *Asp. niger* harvested from the 3-days-shaking culture were resuspended in the medium (2.5 g/50 cc), shaken for described period at 30° and the enzyme activities of the filtrates were determined.

DPG (depolymeric polygalacturonase) was purified by means of the method described on p. 47.

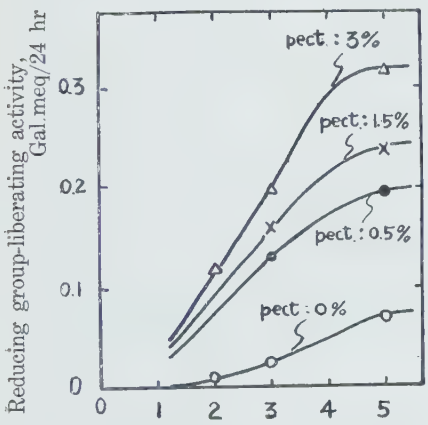


Fig. 3-I. Reducing group-liberating activity in the shaking culture with different proportion of pectin and glucose as C-sources

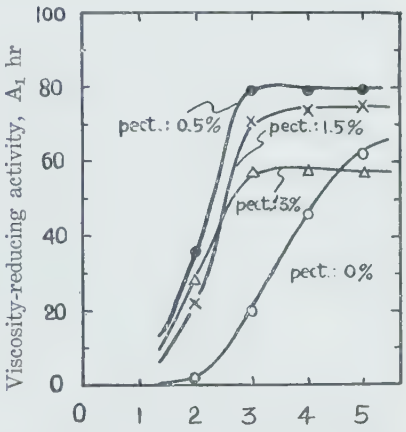


Fig. 3-II. Viscosity-reducing activity in the same culture as Fig. 3-I

The cultures of *Asp. niger* were shaken at 30° with Czapek's salts solution containing pectin and glucose, of which total concentration was 3% in each series. The pH of the cultures were, each day, regulated to 3~5 with a small amount of NaOH or HCl.

Factors influencing DPG-formation. — As seen in Fig. 2 or 3, DPG is formed during the later phase of growth compared to GPG, in the culture without pectic substances. One possible reasen for this phenomenon might be lower solubility of DPG in salt-free water, which delays the exo-secretion of DPG. Since the employed culture medium contains very small quantities of salts at the start, the increase in its ability to extract DPG from the cells must be due to the accumulation of oxalate or other metabolites with the elapsed culture time. But since the exo- and endo-formation of DPG are shown to be fairly parallel (Fig. 4), this delayed extraction of DPG is not considered to be the chief reason for the late elevation of exo-DPG activity. The pH values affecting the formation and the extraction of DPG are somewhat different (Table 2 and 3). The endo-formation is favored at a pH near 2 and exo-secretion is favored at pH 4~5. In the normal course of the cultivation, the pH goes gradually through the above favorable pH ranges. This might be the one reason of the slow appearance of exo-DPG. In Table 2 it is also shown that the presence of

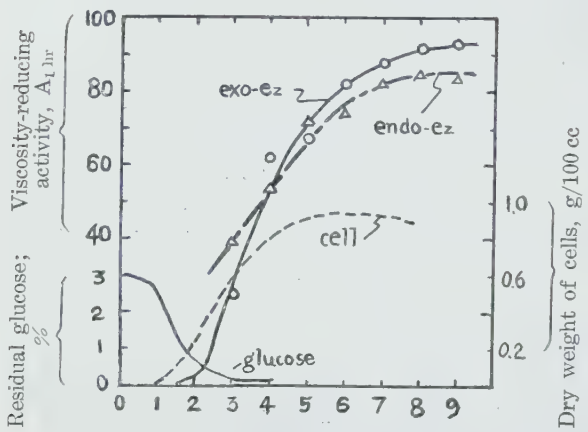


Fig. 4. Exo- and endo-DPG formation

The shaking culture medium was Czapek's salts solution containing 3% of glucose; viscosity-reducing activities of the culture fluid or, for the endo-DPG, of 1% extract of the cells, dried *in vacuo*, with McIlvaine's buffer (pH 4.0), were determined on each day.

Table 2. Effect of pH value on the formation of DPG in rest-shaking of the mold cells

Medium for shaking: McIlvaine's buffer		DPG activity of the filtrate, shaking time: 24 hours
pH	Addition of glucose (0.5%)	$A_{1\,hr}$ (1/8)
2.6	non	34.5
〃	add	37.9
3.6	non	29.6
〃	add	26.9
4.8	non	34.1
〃	add	32.4

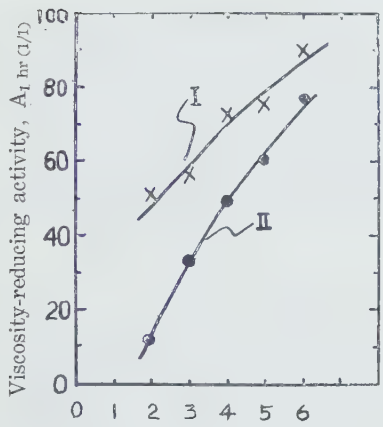
See Table 1, in detail.

Table 3. Effect of pH value of medium for extraction of DPG from the cells

pH value of medium		DPG activity extracted in medium	DPG activity remained in the cells*
Initial	Final	$A_{1\ hr\ (1/1)}$	$A_{1\ hr\ (1/1)}$
1.8	2.4	31.5	24.4
2.0	3.2	38.0	18.1
3.2	3.4	42.7	16.1
3.6	3.6	41.0	14.2
4.0	4.0	45.8	9.9
5.0	4.6	43.3	15.1

Each 2 g of the cells was extracted with 20 cc of the medium comprising 15 cc of 1 M NaCl and 5 cc of M/5 Na_2HPO_4 of which initial pH value was adjusted with HCl, in presence of toluol, at room temperature (18°), for 24 hours. Resulting final pH and the activity of the filtrate were determined.

* Remained activity was estimated with 1% extract of the cells after drying *in vacuo* and grinding.



Period of shaking culture, days

Fig. 5. DPG-formation during rest-shaking

Each 2 g of the mold cells harvested from variously aged shaking cultures were resuspended in 20 cc of McIlvaine's buffer (pH 4.0) and shaken at 30° for 24 hours (curve I), or left standing for 24 hours in presence of toluol at room temperature (18°) (curve II). Viscosity-reducing activities of both subsequent filtrates were determined.

a heterologous substrate, glucose, has no noticeable influence on the enzyme formation. Fig. 5 shows that the rest-shaking of the cells, i.e. shaking without extracellular nutrition, results in the increase of the amount of enzyme as compared with the initial amount of enzyme in the cells, as shown by the activities increased from curve II to I. This was verified by comparing the enzyme activities with extracted enzymes from cells which were dryed *in vacuo* and ground. This fact may suggest that the oxygen supply stimulates the inversion of cellular material, accumulated during growth, to active DPG. Thus the delayed formation of DPG may be partly due to the gradual development of the environmental conditions and due to the fact that it is formed only by the mature cell.

Purification of DPG. — As DPG is formed in glucose-culture independently from other induced pectic enzymes, its purification is easily performed. DPG is seen to be adsorbed by relatively weak adsorbants, poly-

Table 4. Various adsorbants for adsorption of DPG

Adsorbant	pH of DPG solution*1	Ratio of DPG adsorbed to original activity*2	Ratio of DPG eluted to original activity*3
Pulpified filter paper	3.0	71.0%	62.5%
〃 〃 〃	5.4	12.0	11.5
Potato starch	3.0	50.1	48.3
〃 〃	5.4	0	0
Alginic acid	3.0	70.0	32.5
〃 〃	5.4	0	0
Active almina	5.4	57.5	51.6
Fuller's earth	5.4	>98	0
Infusorial earth	5.4	>98	0
Caoline	5.4	>98	<1
Active carbon	5.4	>96	0
Calcium carbonate	5.4	12.8	<1
Konjak mannan	3.0	0	0
〃 〃	5.4	0	0

Each 3% of adsorbant was added to the concentrated and dialyzed DPG solution (66 DPGu/cc), which was left standing, after stirring, for 2 hours at room temperature. The resulting adsorbant was separated by centrifuging from the supernatant which served for determination*2, and eluted with original volume of McIlvaine's buffer (pH 4.0) containing 5% of NaCl for determination*3.

*1 pH of DPG solution was 5.4 and, if necessary, adjusted to 3.0 with HCl.

*2 DPG adsorbed was calculated as to the difference between the original activity and residual activity in the supernatant after adsorption.

saccharides, shown as in Table 4, when the adjusted pH and the salt-free state of the enzyme solution were retained. In these cases elution by salt-solution is quite effective, whereas, from many other general adsorbants, adsorbed DPG can never be eluted. Thus pulpified filter paper, starch, alginic acid, and active alumina are considered to be good adsorbants of dialyzed DPG. Lineweaver and others purified mold polygalacturonase by adsorption on alginic acid[9], but this adsorbant is not easily washable because of its gelatinous nature. Active alumina is undesirable because its adsorbing ability at various pH values is variable according to its state of activity. Starch which has been used for the adsorption of amylase is less specific and it is difficult to pass the filtrate through it, since the starch is very fine. Thus pulpified filter paper is chosen as the most convenient of the adsorbants for DPG purification. DPG is most effectively adsorbed by the pulp from DPG solution at pH 3.8~4.6 (Fig. 6-I). Almost the same range is used when starch is the adsorbant, while the elution of DPG adsorbed onto pulp with a salt-solution of low concentration is effective at a neutral

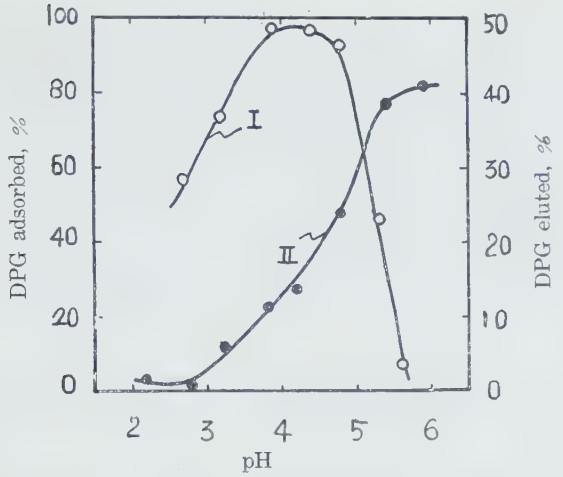


Fig. 6. Effect of pH for adsorption (I), or for elution (II) of DPG

For curve I; each 3% of adsorbant, pulpified filter paper, was added to the dialyzed DPG-solution (35 DPGu/cc, pH was adjusted with minimal amount of HCl or NaOH), which was left standing for 2 hours at room temperature (18°). The ratio of adsorption was calculated from the difference between original and residual activities in the supernatant. The results were confirmed by elution of the adsorbed DPG.

For curve II; each 1.5 g of wet pulpified filter paper adsorbing DPG homogeneously was eluted with 20 cc of water containing 1/4 volume of appropriate buffer, for 2 hours at room temperature, then the supernatant was estimated. The ratio of elution was expressed as percentage to the activity eluted by buffer containing 4% of NaCl, the latter which was almost equal in every pH range (30 DPGu/cc).

Table 5. Effect of salt-concentration for elution of DPG

Medium of elution			DPG eluted*2
McIlvaine's buffer (pH 4.0)	5% NaCl solution	Distilled water	
cc	cc	cc	%
0*1	0	20	37.6
2.5	0	17.5	12.4
5	0	15	43.2
5	2	13	86.5
5	4	11	100.0
5	6	9	96.0

Each 1.5 g of wet pulpified filter paper adsorbing DPG was eluted with 20 cc of medium some of which contained salt, for 2 hours at room temperature; then the activity of supernatant was determined.

*1 The pH value of only distilled water was natural, 5.2.

*2 Each relative effect of elution was expressed as percent activity to the maximum eluted, 8.1 DPGu/cc.

pH (pH 5~6) (Fig. 6-II). When the elution is achieved with highly concentrated salt-solutions the influence of the pH is diminished and elution is easy even at acidic pH's. The effective concentration of salt is more than 1% in the case of NaCl (Table 5).

Precipitation of DPG partially purified by adsorption is shown in Table 6 and 7. The employed concentrations of both the precipitants, ammonium sulfate, and organic solvents, were necessarily high in this case. When organic solvents are used, the DPG solution must be cold in order to get a high percentage of recovery. When unpurified DPG was used, the distribution of DPG activity in each fraction was scattered over a somewhat wider range.

Table 6. Precipitation of DPG by ammonium sulfate

Saturation with $(NH_4)_2SO_4$	DPG activity of precipitate	Recovery	Recovery in each fraction (calculated)
	DPGu/cc	%	%
5/10	5.0	3.4	
6/10	15.0	9.1	5.7
7/10	30.1	18.2	9.1
8/10	89.5	54.2	36.0
9/10	132.0	80.0	25.8
10/10	140.0	84.8	4.8

Appropriate weight of $(NH_4)_2SO_4$ was added in the solution of DPG (165 DPGu/cc, pH 4.0 adjusted with HCl) partially purified by filter-paper-adsorption (described later on), which was left over night in the ice box and, after centrifuging, the precipitate was dissolved in the original volume of distilled water, for the determination of DPG.

Table 7. Precipitation of DPG by organic solvent

Concentration of solvent		DPG activity of precipitate	Recovery	Recovery in each fraction (calculated)
	vol. %	DPGu/cc	%	%
Aceton	40	0	0	
	50	74.5	22.6	22.6
	60	139.2	42.3	19.7
	70	270.0	81.8	39.5
Ethanol	60	59.4	18.0	
	70	210.0	63.7	45.7
	80	218.0	66.0	2.3

Appropriate volume of solvent was added to the solution of DPG (330 DPGu/cc, pH 4.0) cooled with ice bath and, after centrifuging, the precipitate was redissolved in the original volume of distilled water for the determination of DPG.

Table 8. Purification of DPG

*Culture fluid**1 (Czapek's medium, 6~9 days-shaking culture of Asp. niger, pH 2~3)

pH was adjusted to 4.0 with $CaCO_3$;
filtration

↓ Ca-oxalate and Mold cells

↓ *Filtrate*

↓ Residual filtrate

pass through the column of pulpified filter-paper (diam. 5 cm, length 10 cm);

↓ Colored substances

wash out colored substances with N/1000 HCl;

↓ Pulp-column (reuse is possible)

elute with 2% NaCl solution (with 1/5 vol. of pH 5.0-buffer);

↓ *Elute**2

adjust the pH to 4.0 with HCl;
condense *in vacuo* (bathe in 25°)*3;
10/10 saturation with $(NH_4)_2SO_4$,
stand over night in ice box;
centrifuging

↓ Supernatant

↓ *Precipitate*

redissolve in small vol. of water*4;
5/10 saturation with $(NH_4)_2SO_4$,
stand over night in ice box;
centrifuging

↓ Precipitate

↓ *Supernatant*

add $(NH_4)_2SO_4$ to 9/10 saturation,
stand over night in ice box;
centrifuging

↓ Supernatant

↓ *Precipitate*

redissolve in small vol. of water*5;
dialize in cellophane membrane against KH_2PO_4 M/250, 1 day;

↓ *Dialized solution*

add the equal volume of acetone;
centrifuging

↓ Precipitate

↓ *Supernatant*

add the equal volume of acetone;
centrifuging

↓ Supernatant

↓ *Precipitate*

dry in desiccator;

↓ *Purified DPG**6

	*1	*2	*3	*4	*5	*6
Activity, DPGu/cc	200	400	4000	600, 000	700, 000	3000 (/mg)
Recovery, %	100	90	65	50	40	10

DPG produced in the culture with glucose is partially purified by a combination of the above described methods as shown in Table 8. The resultant preparation is stable during the storage at room temperature in a dry state and practically free from amylase and cellulase, both of which had been produced in large quantities in the culture liquid. Other pectic enzymes, pectin methylesterase, GPG, and polymethylgalacturonase[23], are completely absent since they were not produced in the pectin-free culture employed in this study. Activity and recovery, as shown in the table, are somewhat low and should be improved by further study.

Separation of DPG from GPG preparation.—In pectin-containing cultures, many kinds of pectic enzymes are expected to be induced by the substrate[1]. Here the separation of DPG from other enzymes by means of adsorption onto pulpified filter paper was performed (Table 9). Since GPG is also adsorbed though in smaller quantities, the initial fraction of the filtrate showed no glycoside-splitting activity towards pectic acid. In the following fraction, the activity of reducing group-liberation was predominant as compared with the viscosity-reduction activity, and the ratio of these two

Table 9. Fractional adsorption of polygalacturonase-complex

Fraction	Viscosity-reducing activity	Reducing-group-liberating activity	Ratio of $\frac{\text{Red}}{\text{Visc.}}$ *
cc	DPGu/cc	Gal_{meq}/24 hr	$\times 10^3$
0– 50	0	0	—
50–100	13	0.21	16.1
100–150	23	0.36	15.7
150–200	36	0.59	16.4
200–250	51	0.68	13.3
Before absorption	120	0.70	5.8

pH-adjusted culture liquor (pH 4.0) was passed through a pulpified filter paper (5 cm diam. ×5 cm) and collected fractionally.

* Ratio of two activities of preceeding columns qualitatively suggests the ratio of GPG/DPG.

activities remained constant for a while, suggesting the unique composition of the glycoside-splitting enzymes of the filtrate. This fraction was used as GPG free of DPG in the following experiments. When the pulp column was eluted with a salt solution, as previously described, while the ratio was constant, the eluate contained mostly DPG as determined by the high activity in viscosity-reduction. This fact showed that selective adsorption of DPG occurred during the initial stage. Then the pulp colomn was saturated gradually with DPG and, in later fractions of filtrate, GPG was contaminated with DPG as seen in the change of the ratio of two activities. Since no glycoside-splitting activity was recognized in the reaction mixture at pH 6 or higher, even in a pectin-containing culture employed to produce

the polygalacturonase complex, no polymethylgalacturonase[23] was considered to be produced. Amylase or other polysaccharase had been diminished according to the compectitive effect of pectic C-source as reported previously[1]. And even if there was olygogalacturonase-activity, it seemed not to be so strong that it confused the degradation of pectic acid by GPG at the initial stage. Thus such a fraction as obtained by careful fractional adsorption could be used as GPG practically free of other enzymes when it acted on pectic acid (in order to neglect the pectin methylesterase-action, which was seen to comtaminate the above preparation), though the total picture of the following experiments may not necessarily represent the single enzyme action of GPG.

Properties of DPG and GPG. — By means of viscosity measurements the optimum pH of DPG action is found to be 3.5 when the substrate is pectin, which is already known as the value of the optimum pH of fungal polygalacturonase[8,10]. In the case of pectic acid, a fairly high pH value, 4.8, favors its action, nevertheless no appreciable spontaneous change of viscosity of pectic acid solution that might suppress the enzyme action is recognized even in such acidic pH ranges as shown in the figure (Fig. 7).

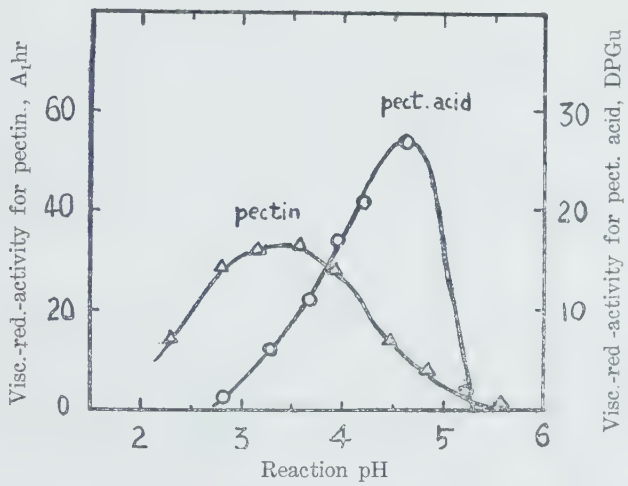


Fig. 7. Optimum pH of DPG action on pectin or pectic acid by viscosity-measurement

Each pH value was controlled by adding the equal volume of appropriate buffer to the 2%-substrate-solution. DPG of 21 DPGu/cc was used.

When the liberation of reducing groups from pectic acid are estimated, the optimum pH for DPG action lies almost in the middle of the two values (Fig. 8), and it seems that the less the reaction time the higher the apparent optimum pH, a tendency which might be due to the stability of DPG at a pH near 3.5 (Fig. 9), or to the fact that the enzyme action has different optimum pH's towards the substrates of different molecular sizes.

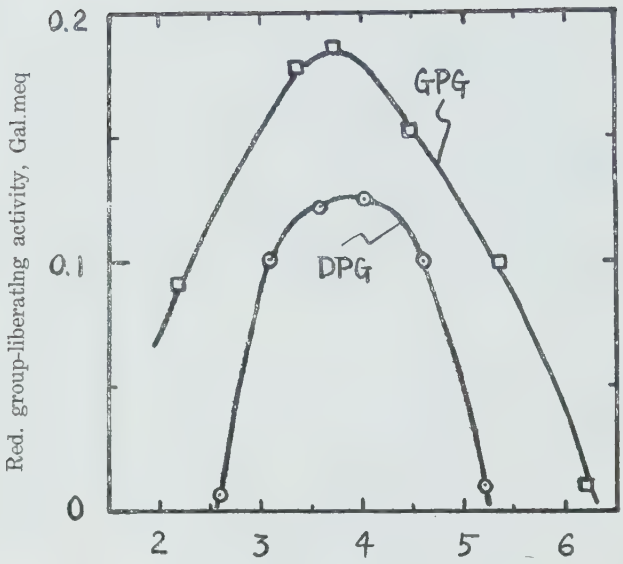


Fig. 8. Optimum pH of DPG or GPG on pectic acid by reducing group-mesurement

Increase of liberated reducing groups for 24 hours (for DPG) or 21 hours (for GPG) by 1 cc of either enzyme solution (21 DPGu of DPG or GPG described on p. 48~49) added to 20 cc of 1% of pectic acid solution buffered by half a volume of appropriate buffer, was estimated and expressed in milliequivalents.

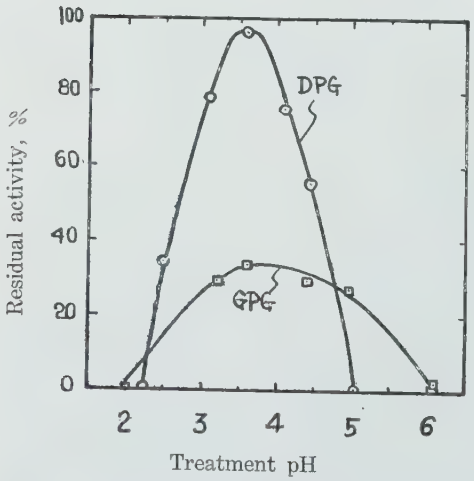


Fig. 9. Stability of DPG or GPG with pH

1 cc of purified DPG solution (200 DPGu) or GPG was treated with the equal volume of McIlvaine's buffer of treatment pH at 50° for 15 min. (for DPG), or for 1 hour (for GPG); then 1 cc of treated DPG was immediately diluted in 2 cc of cold buffer (pH 4.2), 1 cc of which was put into 1% substrate solution; GPG was directly put into the substrate solution buffered at pH 4.2, both were then incubated at 30°; The residual activity was expressed as percent activity to that not treated (33 DPGu, or 0.92 Gal.meq/21 hr for GPG).

The reaction pH of GPG towards pectic acid has a peak at pH 3.5~4.0, similar to the last case of DPG, but over a wider range (Fig. 8), a tendency which is also observed in stability curves of the enzymes with altered pH values (Fig. 9) or, in other words, GPG is fairly stable and active even at neighbor pH's 5 or 2 while DPG is not.

The stability of both enzymes when heated are shown in Table 10. Reductions in half the activity are observed at 50° for DPG and at 60° for GPG when heated for 1 hour at pH 3.6, the latter appearing to be more stable. However, the purity of the GPG preparation was so much less than that of DPG, that it is possible that these apparent differences of stability might be affected by the impurities.

Table 10. Inactivation of enzymes with temperature

Temperature of treatment	DPG		GPG	
	Residual activity	Rate of inactivation	Residual activity	Rate of inactivation
C°	DPGu/cc	%	Gal_{meq}/20 hr	%
no —	23	—	0.201	—
30° for 1 hr	23	0	0.202	0
40° for 1 hr	23	0	0.189	5
50° for 1/2 hr	15.7	32	—	—
50° for 1 hr	10.3	55	0.152	24
60° for 1/2 hr	<1	>96	—	—
60° for 1 hr	—	—	0.098	51
70° for 1 hr	—	—	0.002	98

The enzyme solution was maintained at pH 3.6 during the treatment.

Table 11. Influence of salts on activity of enzymes

Salt added		Apparent activity of DPG		Apparent activity of GPG
	Concentration	Viscosity-reducing activity	Reducing-group-liberating activity	Reducing-group-liberating activity
	N	DPGu/cc	Gal_{meq}/5 hr	Gal_{meq}/20 hr
non	—	40	0.045	0.229
NaCl	0.2	34	0.038	0.263
〃	0.1	48	0.049	0.250
〃	0.05	—	—	0.250
〃	0.025	—	—	0.244
KCl	0.1	48	0.051	0.220
〃	0.05	—	—	0.252

The pH value of substrate solution without buffer was adjusted to 4.2 for DPG or to 3.8 for GPG with minimal amount of NaOH (the final concentration is N/100~N/200).

It has been recognized that many metallic ions promote the polygalacturonase-action[8,10]. Here only feeble but similar effects of salts were observed in the case of DPG and GPG (Table 11), though there was again a slight difference between the two enzymes. Since salts of polyvalent metals cause the coagulation of pectic acid, the stimulating effects of these salts were recognizable to be somewhat higher than monovalent salts only when pectin was used as the substrate in order to avoid the coagulation.

DPG seems to split the glycosidic bonds which are free from methoxyl radicals as has been known in the case of polygalacturonase[6]. This fact is shown by negligible liberation of reducing groups from pectin though the viscosity reduction is almost the same as in the case of pectic acid (Fig. 10). The preceding demethoxylation of pectin by sodium hydroxide is seen to increase the extent of reducing group-liberation in proportion to the quantity of alkali employed in the demethoxylation treatment. The same experiment is unsuccssful when GPG is employed because of the presence of pectin methylesterase.

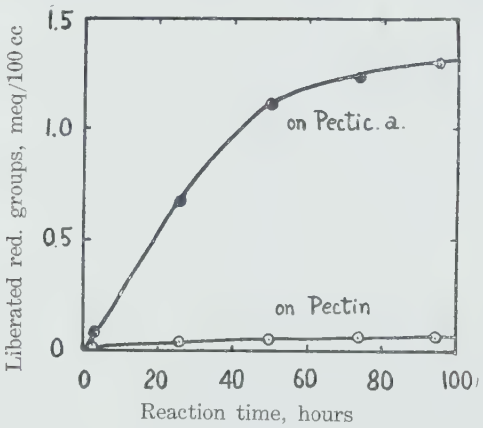


Fig. 10. DPG-action on pectic acid or pectin
1 cc of purified DPG solution (50 DPGu) was added to 50 cc of 1% substrate solution (pH 4.2); pectic acid containing 68% uronic anhydride, and citrus pectin containing 65% uronic anhydride and 7.8% methoxyl. The reaction was carried out in the presence of toluol at 30°, and the liberated reducing groups were determined.

The kinetics of polygalacturonase action towards pectic acid has been known to occur in two steps[6]: the first stage, a zero order reaction having the Km as low as 0.03%, proceeds until 45~50% of the pectic acid reducing groups are hydrolyzed and the second phase is of 1/15 slower velocity in which the Km is greater than 1%, resulting in complete hydrolysis.

The appearance of such a clear two step-reaction was not recognizable in either the case of DPG or GPG as seen in Fig. 12 or 14, or even in shorter periods of the polygalacturonase action although the degree of accuracy is low. Moreover Jansen's suggestion that the substrate in the second phase might be digalacturonic acid or other intermediate products degraded during the first stage of the reaction, was not true in the present case as shown by paper-chromatography (Fig. 13 and 15). Even with purified DPG the relationship between the reaction velocities and the substrate concentrations varied with the time of reaction (Fig. 11), showing that the reaction might occur in one step and that it is not zero- but rather a first-

order reaction. At all events, the kinetics of pectic degradation remains a matter of speculation as long as the accuracy of determination of the enzyme activity is not improved, as many investigators have pointed out.

Modes of action. — Obvious differences between these two enzymes were observed in the modes of action on pectic acid as shown in Fig. 12~15. Since quantities of both enzymes were controlled to produce the same reduction in the viscosity of the substrate in the cases of Fig. 12 and 14, apparent differences in the rates of reducing-group-liberation could be observed. DPG slowly splits the glycosidic bonds, and the extent of hydrolysis hardly exceeds 50% of the original uronic acid content. As shown in Fig. 13, the initial products of DPG-action are polygalacturonic acids remaining at the original spot but they lose their coagulability in acid during the first four hours, then eight hours after starting time tetra-, tri-, di-, and mono-galacturonic acids are produced and the extent of hydrolysis is only 8%. When the hydrolysis reaches about 50%, only tri-, di-, and mono-galacturonic acids are left as end products which hardly disappeared even when the reaction mixture was twice fortified with DPG 168 hours after the start of the reaction and the reaction continued. In contrast, GPG rapidly liberates only mono-galacturonic acids and the hydrolysis easily goes to completion. However, the molecular size of the substrate, most of which remains at the original spot, slowly decreases. This is shown by precipitation with acid: the substrate loses its acid-coagulability much more slowly than with DPG (Fig. 13 and 15). Olygogalacturonic acids barely appear after 48 hours where 65% hydrolysis has been completed in contrast to the earlier appearance of oligogalacturonic acids by DPG action, and these spots redisappear rapidly. The extent of hydrolysis at the point of half reduction of viscosity is 7% or higher, while in the case of DPG, it is 3% or less, the latter being similar to the already known value of polygalacturonase[6]. Thus it is suggested that DPG attacks glycosidic chain at random producing oligogalacturonic acid but not hydrolyzing tri- or di-galacturonic acid, and that the end-products of this reaction are the mixtures of these two and mono-galacturonic acids, whereas the GPG splits galacturonic acids one by one from one end of glycosidic chains with the slower reduction of the substrate molecular size.

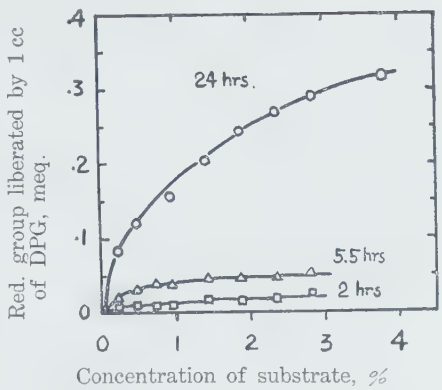


Fig. 11. Influence of substrate-concentration on the action of DPG

Pectic acid-solution of appropriate concentration was incubated at 30° with DPG of 25 DPGu for 24 hours, or of 37.5 DPGu for 5.5 or 2 hours.

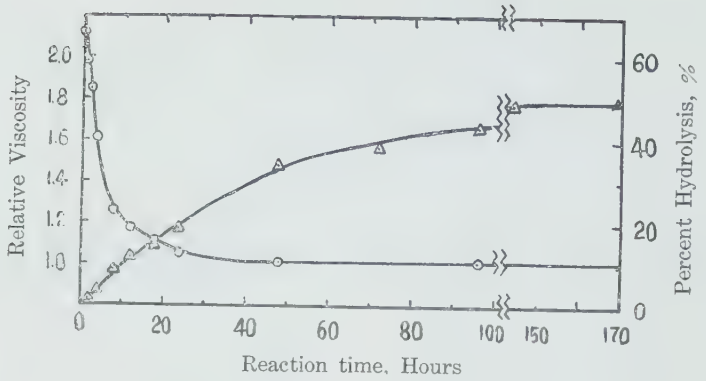


Fig. 12. Action of DPG on pectic acid

600 DPGu of DPG was added to 500 cc of 1% pectic acid solution (pH 4.0) and reacted in 30° incubator in the presence of toluol. Each aliquot of subsequent reaction mixture to serve for analysis was inactivaed by heat. Percent hydrolysis was based on total uronic acid content (68% of pectic acid).

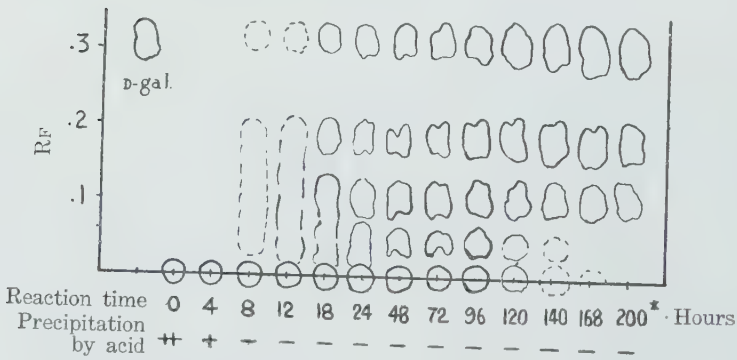


Fig. 13. Paper-chromatogram of reaction mixture during the course of DPG action of Fig. 12.

0.3 cc of the heat-inactivated reaction mixture was developed on TOYO-Roshi's No. 50 filter paper with solvent: n-buthanol: acetic acid: water=5:2:3. The spots were revealed by aniline-phthalate at 100°,15 min. Precipitate formation by addition of 1/5 vol. of 1 M H_2SO_4 to the reaction mixture means as follows: ++: coagulated, +: finely precipitated, ±: turbid, −: clear. Left control spot is of D-galacturonic acid.

* The spots on 200 hours show the limit of hydrolysis even with twice fortified DPG added at 168 hours.

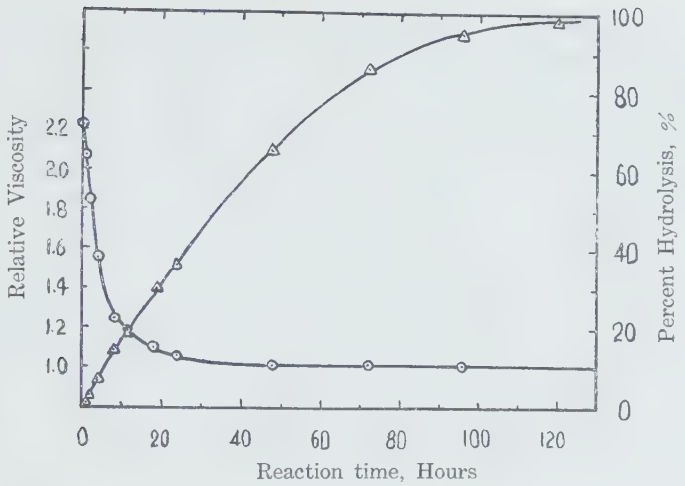


Fig. 14. Action of GPG on pectic acid

50 cc of GPG solution was added in the reaction mixture; other conditions were the same as Fig. 12.

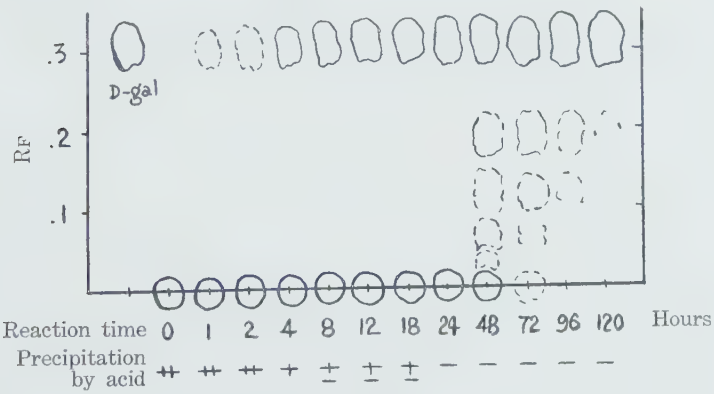


Fig. 15. Paper-chromatogram of reaction mivture during the course of GPG action of Fig. 14.

If these modes of action are assumed a reasonable explanation is obtained for the phenomenon described above and for Table 12 and 13. Polygalacturonic acids partially pre-hydrolyzed by DPG are split by GPG at a higher rate than the original pectic acid when the extent of pre-hydrolysis is not great enough to decrease the affinity of GPG for the substrate too much (Table 12). This may be explained by assuming that terminal groups increase and allow themselves to be split by GPG, according to the random attack of DPG. Since the Km of GPG acting on slightly lower polygalacturonic acids is comparatively high, this is a possible explanation. Nevertheless, when DPG and GPG co-acted on pectic acid

Table 12. GPG-action on polygalacturonic acid pre-treated by DPG

Pre-treated polygalacturonic acid			Increase of reducing group by GPG-action for 24 hours	Hydrolysis rate per one molecule of substrate*
Time of pretreatment by DPG	Percent Hydrolysis	Reducing group		
hour	%	meq/100 cc	meq/100 cc	
no	0	0.34	0.42	1.24
4	3.2	0.45	0.57	1.27
10	9.6	0.68	0.78	1.15
36	27.0	1.19	0.86	0.72

1% pectic acid solution (pH 4.0) was pre-treated by DPG as in Fig. 12. After heat-inactivation of DPG, 1 cc of GPG solution was added to this solution and reacted at 30°.

* The ratio of increased reducing groups by GPG to initial reducing groups after pre-treatment.

Table 13. Influence of co-action of DPG and GPG on the hydrolysis of pectic acid

Mixture of enzymes	Liberated reducing groups as milliequivalent in 100 cc of reaction mixture during:	
	5 hours	24 hours
GPG	0.28	1.31
DPG	0.12	0.64
GPG + DPG	0.44	1.85

Either or both of GPG solution (1 cc) and DPG (20 DPGu) were added to 20 cc. of 1% pectic acid solution (pH 4.0) and reacted at 30°.

simultaneously, no complementary effects of both were observed, but only the sum of the activities were observed (Table 13), a fact which seems to reveal the independent action of both on pectic acid, i.e. it is probable that GPG is also one of pectic polygalacturonases which acts on pectic acid itself without any precedent degradation. If there was any slight increase of GPG activity resulting from the action of DPG, it might have been hidden by the independent activities or suppressed by the adhesive nature of pectic acid substrate in the case of Table 13.

DISCUSSION

Ever since Ehrlich had postulated the existence of pectinase and pectolase in his classic study, many investigators have assumed that hydrolysis of polygalacturonic acid consists of two steps, i.e. polygalacturonase and oligogalacturonase split the substrate stepwise[5,6,15].

On the other hand, according to the information on fungal polygalacturonase, other types of pectic glycosidases acting on polygalacturonic acid have been found to be derived from different sources. McColloch and Kertesz's tomato-depolymerase[13], Luh and Phaff's yeast polygalacturonase[11], Seegmiller and Jansen's polymethylgalacturonase[23] or Roboz and other's pectin depolymerase[19], and Ozawa's polygalacturonase II of caarot[16] are some of these types of pectic glycosidases.

Recently Schubert has studied the enzyme complex of *Aspergillus niger* by means of inactivation or adsorption techniques, and differenciated four types of glycosidases, one of which is presumably corresponds to polymethylgalacturonase but the others remain in obscurity as concerns their modes of action[22]. Jermyn and Tomkins[7] using paper chromatography to study the course and the products of the reaction, revealed the probability of random scission of polygalacturonic acid occuring rapidly to about 50%-hydrolysis, but finally and slowly ending in complete hydrolysis accompanied by the accumulation of D-galacturonic acids. Similar results were also obtained rescently[14]. However, if the purification of polygalacturonase preparation is performed only in terms of reducing group liberating activity as seen in some reports, it seems to be impossible to obtain the enzyme which incompetely hydrolyzes pectic acid and, as a result, to analyse the composition of the enzyme-preparation. Ayres *et al.* and Dingle *et al.*[2,3] analyzing mold enzyme preparations, postulated that such crude preparations contained at least three pectin-splitting enzymes. One seemed to be polymethylgalacturonase, and the other two, one : an enzyme which converts pectic acid to tri- and di-galacturonic acids, and two : an oligogalacturonase which hydrolyzes only the latter two acids to monogalacturonic acids. It is partially impossible that such an explanation favors the theory presented in this paper. Some of the classical information on polygalacturonase studied was obtained with crude preparation and therefore might have been dealing with the "polygalacturonase-complex", but it is the contemporary belief that there may be a considerable number of types of pectic glycosidases acting on pectic polygalacturonic acid already demethoxylated.

In the present work with enzymes of *Aspergillus niger*, two types of polygalacturonases are recognizable from the point of view of the factors affecting enzyme formation and of the mode of action. In the former, the induced enzyme formation is partially coincidental with the results of a study by Gäumann *et al.*[4] in which it is suggested that the formation of polygalacturonase is simply constitutive ; and with Luh and Phaff who recognized constitutive formation of yeast polygalacturonase[11]. One of the polygalacturonases described in the present paper, DPG, purified from the culture liquor which is free of pectic substances and completely free of other induced pectic enzymes, has similar properties to already known fungal polygalacturonases in many respects, but this cannot hydrolyse tri- and di-galacturonic acids, at least practically, thus leaving these two and

mono-galacturonic acids as end products in the final reaction mixture in which 50% of the pectic acid was hydrolyzed. The latter activity is similar to polymethylgalacturonase activity[23], but the optimum pH of DPG though variable with substrate quality and reaction period, being 3.4~4.6, is obviously different from polymethylgalacturonase. Yeast polygalacturonase[11] or polygalacturonase I of *Rhizopus* producing tri- and di-galacturonic acids as intermediate products[16] may be a related enzyme. If further assumptions are made, some of the previously investigated fungal polygalacturonases might be the mixed preparations comprising of DPG itself and a little oligogalacturonase and/or GPG remaining even after the purification, because the latter may be induced in abundant quantities under the culture conditions employed in those studies.

On the other hand, GPG, induced by the presence of pectic substrate in the culture of *Aspergillus niger*, if free of DPG, shows the appearance of the reaction which is different from the other already known fungal polygalacturonases. Polygalacturonase II derived from carrot by Ozawa[16] has a similar mode of action but different properties. Saccharifying polygalacturonase extracted from mycelia of *Penicillium expansum* harvested from pectic acid-culture, reported by Ozawa and Okamoto[17], may be more closely related to GPG. Though there is some possibility that the present GPG-preparation may contain oligogalacturonases, or small amounts of DPG, it is fairly certain that the enzyme termed as GPG attacks the pectic polygalacturonide, splitting the terminal galacturonic acids without the pre-degradation of the substrate by DPG or other enzymes.

The extents of enzymatic hydrolysis of polygalacturonic acids with altered molecular sizes have been studied in some detal. Matus[12] demonstrated that the extent of hydrolysis of polygalacturonic acids previously degraded enzymatically was greater with high-molecular-weight substrates than with the lower ones, but McCready and McComb[14] recognized no difference in the extents with acid-degarded substrates. Ozawa and Okamoto[18] observed considerable inconsistencies in such conclusions due to the variety of pre-decomposing agents or pectic enzymes. In the present work the rate of GPG action on polygalacturonic acids slightly pre-degraded by DPG was somewhat accerelated, though no increase of the extent of hydrolysis could be observed. However, this is a problem related to the kinetics of enzyme action or the purity of enzyme preparation and the correct analysis may be difficult without more careful techniques as pointed out previously, thus leaving some quetion as to the size limit of the molecules that GPG can act upon.

SUMMARY

Pectic glycosidases produced by a strain of *Aspergillus niger* comprise at least two kinds of polygalacturonases, each of which is formed in different ways depending on the culture conditions, especially on the kinds

of C-sources. The one, depolymeric polygalacturonase (DPG), produced constitutively by the mold, attacks the polygalacturonide chain at random, reducing the molecular size rapidly, and leaving tri-, di- and D-galacturonic acids as end products in the final reaction mixture where more than 50% of the substrate is hardly hydrolyzed. This enzyme was effectively purified by adsorption onto pulpified filter paper, followed by the general method of precipitation, from the culture liquor which did not contain pectic substrate and other adaptive pectic enzymes. DPG is easily adsorbed in a salt-free state at a pH near 4 and eluted with a more neutral salt-solution. The reaction pH has the optimum value of 3.4~4.6, variable with the substrate and with the method of determination. DPG is most stable at pH 3.6, where the half activity is reduced by treatment at 50° for 1 hour. This enzyme seems to hydrolyze the non methoxylated glycosidic chain. The another polygalacturonase, galacturonogenic polygalacturonase (GPG), the formation of which is induced by the pectic substrate in contrast to DPG, splits the terminal galacturonic acid from one end of the polygalacturonide chain, not reducing the molecular size of the substrate as rapidly as DPG does. Other properties of GPG are similar to those of DPG though there are some differences which might be due to the lesser purity.

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BERIBERI-LIKE SYMPTOMS IN MICROORGANISMS

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A Brief Description of the Phenomenon

The most representative symptom, which commonly appears as a result of vitamin B_1-deficiency, is a neuritis known as beriberi. Beriberi is only a symptom or phenomenon which appears on the outside, but the inner disorder causing this phenomenon has not yet been fully clarified. However, it is well known that this symptom can be cured by administering vitamin B_1.

Though the chemical structure of vitamin B_1 has been clarified and its mass-production by chemical synthesis has also been established, the reason why beriberi can be cured with vitamin B_1 still remains obscure.

We must remember, however, the following two epochmaking findings concerning this problem, namely: (1) Peters, Thompson and Johnson found that vitamin B_1-deficiency in food lowers the vitamin B_1 level in blood and causes an increase in the pyruvic acid content, and (2) Lohmann and Schuster claimed that cocaroxylase is a pyrophosphoric ester of vitamin B_1.

It may be a matter of course that the vitamin B_1 level lowering in blood is parallelled by its deficiency in the food, but it is very interesting to us that pyruvic acid is accumulated abnormally in this case.

As pyruvic acid occupies a central key-position not only in carbohydrate metabolism but also in almost every important metabolism, it must be biochemically very unstable. Accordingly, the accumulation of pyruvic acid can hardly be seen in normal conditions. In yeast fermentation, for example, all the sugar molecules change to alcohol and carbon dioxide via pyruvic acid, but even mere detection of this intermediate substance is said to be very difficult. Here, if anyone could create the appropriate conditions to make yeast accumulate pyruvic acid without retarding its growth too much, it appears reasonable to say that yeast would suffer from "beriberi". But we have not yet heard of such results; perhaps this is because yeast can synthesize vitamin B_1 and has sufficiently strong carboxylase activity.

There are several strains in moulds, especially in *Phycomyces*, *Fusarium*, *Mucor*, and *Rhizopus* which lack the ability to synthesize vitamin B_1, and have carboxylase whose characteristics are somewhat different from those of yeast-carboxylase. Among the moulds *Mucor mandshuricus* Saito is the most conspicuous and we can see remarkable beriberi-like symptoms in this species.

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Table 1. Biochemical characteristics of the members of *Mucor* group

	Sub-group	Jodoform reaction	Succinic acid	Lactic acid	Other oxy-acids	Fumaric acid	Ethanol
M. alpinus Hansen		±	+	−	−	−	++
M. alternans (−)	C	++	−	−	−	−	+
M. botryoides var. minor Jensen		+	+	−	−	−	±
M. christianiensis Hagem	No growth in the experimental conditions						
〃 〃	Ditto.						
M. circinelloides van Tieghem	C	++	+	−	−	−	+
M. cunninghamelloides Pispek		−	+	−	−	−	++
M. dimorphosporus Lendner	C	+++	−	−	−	−	++
〃 〃	C	++	−	−	−	−	++
M. dubius Wehmer (+)	C	+++	−	−	−	−	++
〃 〃 (−)	C	++	+	−	−	−	++
M. electus Bainier (+)	R	++	−				++
〃 〃 (+)	R	++	−	+	±	−	++
M. fuscus Bainier		−	+	±	±	−	+
M. glomerula (Bainier) Lendner		±	−	−	−	−	++
M. griseocyanus Hagem (+)	C	−	+	−	−	−	+
M. hiemalis Wehmer (+)	M	−	+	−	−	−	+
〃 *var. Toundrae* Lendner	M	−	+	±	−	−	++
M. Janseni Lendner	C	++	−	+	−	−	++
M. Janchae Lendner		++	+	−	−	−	++
M. javanicus Wehmer (+)	C	++	+	±	−	−	++
〃 〃 (+)	C	++	−	±	−	−	++
〃 〃 (−)	C	++	+	±	−	−	++
〃 〃 (+)	C	++	−	±	−	−	++
〃 〃 (−)	C	++	−	+	−	−	++
〃 A 34	C	++		±	−	−	++
M. lamprosporus Lendner	C	+	−	+	−	−	++
M. mandshuricus Saito	C	+++	−	(+)	−	−	++
M. plumbeus Bonorden	C	±	+	±	−	−	++
〃 〃 (Luft)	C	++	+	−	−	−	+
M. Praini Chodat et Nechitch	C	+	+	−	−	−	++
M. pusillus Lindt	R	−	+	−	−	−	+
〃 〃 2	R	−	+	−	−	−	+
〃 C	R	−	+	−	−	−	+
M. racemosus Fresnius (+)	R	+	+	−	−	−	++
〃 *var. X* Saito et Naganishi	R	+	+	−	−	−	++
〃 A 33	R	+	+	−	−	−	++
M. Rouxii Wehmer (Amyloyces)	C	++	+	−	−	−	++
〃 〃 (〃)	C	−	+	−	−	−	++
〃 〃 (〃)	C	+	+	−	−	−	++
M. Spinensis	C	+		−	−	−	++

Note: C=Cymomucor, R=Racemomucor, M=Monomucor
(After original descriptions)

The strain used in this investigation was first isolated from Manchurian "chiizu" by K. Saito and H. Naganishi and preserved at Takada Institute for Fermentation as *M. mandshuricus 4574*. The present authors selected this strain out of 41 strains of *Mucor* as the most powerful pyruvic acid accumulator without any other detectable miscellaneous fermentations.

Formation of pyruvic acid by *Mucor* was first discovered by S. Kato in 1923, and later confirmed by T. Takahashi and T. Asai. In the course of a study on lactic acid formation by the *Rhizopus*-group, we found the abnormal pyruvic acid accumulation in *R. salebrosus*. We were interested in this phenomenon and we selected the most convenient strain from the *Mucor*-group. Pyruvic acid accumulation can be considered as one of the general properties of *Mucor*-group, and is especially distinct in the strains belonging to Cymomucor (Table 1).

By using *M. mandshuricus* the authors were surprised by a very distinctive phenomenon: in glucose-mineral medium as much as 90 mg of pyruvic acid was formed in 50 ml of the medium, while in malt extract no detectable quantity of the acid was produced (Table 2).

Table 2. Comparison of synthetic medium and malt extract on pyruvic acid accumulation

(Synthetic medium: glucose 5%, NH_4NO_3 0.2%, KH_2PO_4 0.1%, $MgSO_4 7H_2O$ 0.05%, $CaCO_3$ 5%. Malt extract: Brix 7°, $CaCO_3$ 5%. Culture: 50 ml, 30°C, 7 days.)

	Synthetic medium	Malt extract
Initial sugar mg	2,450	2,810
Sugar consumed 〃	1,916	2,102
Pyruvic acid 〃	88	trace
Ethanol 〃	540	664
Dried pellicle 〃	70	80

Since this strain carries on, like other moulds, aerobic respiration besides alcoholic fermentation, the sum of the products in the culture solution cannot be so large. But still the main product is ethanol (ca. 30%) and almost no other substance can normally be fixed.

Comparing the two media, we cannot see any differences in sugar consumption, pellicle formation, and alcohol production. Hence, we can point out that the pyruvic acid accumulation is quite akin with the abnormal increase of pyruvic acid in animal blood when it gets beriberi.

Mould-"Beriberi" and Vitamin B_1

Experiments were carried out to see whether the pyruvic acid accumulation of the mould can be depressed by vitamin B_1 addition. Table 3 show the results of experiments when *M. mandshuricus* was cultured on the glucose-salt media with different amounts of vitamin B_1.

Table 3. Correlation between vitamin B_1 and pyruvic acid-accumulation in *M. mandschuricus*

Glucose 2,000 mg, 50 ml, 6 days

Vitamin B_1	μg	2.5	0.25	0.025	0
Sugar consumed	mg	1,280	860	943	1,050
Pyruvic acid mg	Observed	0	51	71	90
	Calculated	0	50.3	72.6	90
Ethanol	mg	264	215	215	264
Dried mold-pellicle	mg	100	90	90	100

Minute amounts of vitamin B_1 depress the accumulation of pyruvic acid, and if the quantity of the vitamin B_1 exceeds a certain limit, pyruvic acid can no longer be detected.

Considering this, the previous results obtained from the malt extract may be attributed to the inclusion of vitamin B_1. In other words, the pyruvic acid accumulation of *M. mandshuricus* may be concluded as being nothing but a B_1-deficient symptom, viz. "beriberi".

The authors have been investigating this phenomenon since 1950, and the results, entitled as *Pyruvic Acid Fermentation*, were published one by one in the *Journal of Fermentation Technology*. The ultimate purpose of this research is to find out the cause of this microbiological phenomenon and to contribute some information concerning the B_1-deficient symptoms of man and animals which appear to be difficult to solve because of their complex physiological organization.

Functional Relationship between Dosage of Vitamin B_1 and Degree of "Beriberi"

We saw that the accumulation of pyruvic acid is depressed if *M. mandshuricus* was cultured in the presence of vitamin B_1. Naturally there must be a relationship between the quantity of vitamin B_1 added and pyruvic acid produced. In Table 3 "beriberi" (pyruvic acid accumulation) can never be seen at the dosage (concentration of vitamin B_1) of 5 μg% (2.5 μg/50 ml) or more, but at the dosage of 0.5 μg%, about half the puruvic acid is formed compared to the control experiment without vitamin. Accordingly, the limiting concentration of vitamin B_1 which can just stop the accumulation of pyruvic acid must lie between 0.5 and 5 μg%, and this has been shown as being about 2 μg% by repeated experiments.

Higher animals must also have the proper minimal B_1 demand determined for each species, but they live by moving freely and the vitamin taken is excreted in due time. Accordingly, unlike microorganisms in flask, their demand must be determined by day-by-day dosages.

If an animal cannot get its requisite amount of vitamin B_1, it will

reveal more or less the beriberi symptom, but it is not so easy to determine the minimal demand. For example, 600 μg per day per head or 10 μg per kg of body weight, determined as being the minimal necessary dose for adult man, is an average value obtained after long labourious and expensive experiments. Furthermore, the functional relationship which naturally should exist between the insufficient dosage of vitamin B_1 and severeness of the symptom, can hardly be determined.

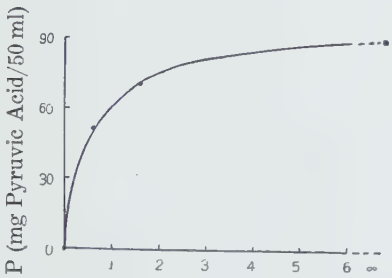


Fig. 1. Functional relationship between vitamin B_1 and Pyruvic acid

In microorganisms, on the contrary, its minimal demand can be determined within several days, and the functional relationship, if desired, can also be found easily by repeating experiments mentioned above (Fig. 1).

Examination of the effect of a large excess of vitamin B_1 may also not be meaningless. In *M. mandshuricus*, no harmful effect was detected at the concentration of 500 μg and 1 mg per 50 ml, namely, 500–1000 times larger than the critical demand for normal metabolism. But we do not know whether there is any data in medical literatures, wherein as much as 1 g of the vitamin was administered every day.

The cause for the decrease in the accumulation of pyruvic acid upon the administration of vitamin B_1 can seemingly be recognized by the structural relationship of the vitamin to cocarboxylase. However, the results of experiments up to the present have some questionable points which cannot be explained by the above relationship. The authors will refer to this point later again.

Other Active Substances which Depress the Pyruvic Acid Accumulation

1. Pyrimidine and thiazole moieties of B_1:— The pyrimidine moiety (2-methyl-4-amino-5-hydroxymethyl-pyrimidine) has been shown to prevent *M. mandshuricus* from getting "beriberi" while the thiazole moiety (4-methyl-5-β-hydroxyethyl-thiazole) has no specific effect even at higher concentrations (Table 4 (a)).

The minimum concentration of the pyrimidine which can completely prevent the accumulation of pyruvic acid, and the concentration which depresses the accumulation to 50%, are presumed to be from Table 4 (b) 4 μg% and 0.6 μg% respectively. Although these values are slightly larger than the corresponding values of vitamin B_1, we can consider the pyrimidine as having about the same order of activity as vitamin B_1. This moiety appears to act as a precursor of B_1, while thiazole can be

Table 4 (a). Effect of pyrimidine and thiazole moieties of B_1. glucose 1,590 mg, 50 ml, 4 days, 30°C

		Pyrimidine 1 mg	Thiazole 1 mg	Pyrimidine 1 mg + Thiazole 1 mg
Sugar consumed	mg	720	540	845
Pyruvic acid	〃	trace	54	0
Ethanol	〃	167	60	258
Dried mold pellicle	〃	60	60	60

Table 4 (b). Limiting concentration of pyrimidine moiety of B_1. glucose 1,490 mg, 35 ml, 6 days

Pyrimidine	μg	0	0.1	0.3	0.6	2.4
Sugar consumed	mg	865	1120	968	1,120	1,150
Pyruvic acid	〃	103	71	42	26	trace
Ethanol	〃	218	310	254	305	259
Dried mold pellicle	〃	65	80	80	75	70

snythesized freely by *M. mandshuricus*. But, if the pyrimidine has B_1-like activity by itself, the synthetic ability towards thiazole becomes out of the question.

These results are somewhat different from the observations on human beings. Since the antiberiberi activity of vitamin B_1 is destroyed by the action of a hydrolytic enzyme called aneurinase, both the pyrimidine and thiazole moieties could not have any effect against human beriberi.

2. Amino acids, substances of vitamin B_1-group, and nucleic acid:— Several amino acids (α-alanine, β-alanine, leucine, glutaminic acid, asparagine, arginine, histidine, methionine, cysteine), urea, acethylcholine, and vitamins of the B-group such as B_2, B_6, folic acid, nicotinic acid, *p*-aminobenzoic acid, pantothenic acid, inositol, and biotin, were tested singly and in combinations, and were shown to have no effect on pyruvic acid accumulation except vitamin B_2 (Table 5 (a)).

Table 5 (a). Effect of substances of the Vitamin B group at higher concentrations (glucose 1,490 mg, 35 ml, 5 days)

		Folic acid 1 mg	Nicotinic acid 1 mg	PABA 1 mg	Pantothenic acid 1 mg	Inositol 1 mg	Biotin 1 μg	B_6 1 mg	B_2 1 mg	O
Sugar consumed	mg	585	565	490	750	515	686	660	732	655
Pyruvic acid	〃	42	56	49	68	54	71	33	**trace**	63
Ethanol	〃	109	100	98	178	86	158	57	162	132
Dried mold-pellicle	〃	60	90	50	55	60	90	30	40	60

The activity of vitamin B_2 is weaker than vitamin B_1, and at concentrations of 18 and 40 $\mu g\%$, the pyruvic acid accumulation is depressed 50 and 100 % respectively (Table 5 (b)).

Table 5 (b). Effect of vitamin B_2
(Initial glucose 1,490 mg, 35 ml, 7 days)

B_2	μg	0	5	12.5	25	B_1: 0.27 γ
Sugar consumed	mg	1,110	1,225	1,010	960	1,084
Pyruvic acid	〃	104	67	9	trace	trace
Ethanol	〃	202	236	124	144	248
Dried pellicle	〃	80	90	70	60	110

It is very interesting to note that this result coincides with the result of I. Nakagawa obtained by human beings. According to him if the administration of vitamin B_2 was insufficient, the pyruvic acid content of blood markedly increased just as in the case of B_1-deficiency.

"Polypeptone" (Takeda) was shown to have some effect against pyruvic acid accumulation. By adding 10 mg% of polypeptone, pyruvic acid accumulation decreased to about 50 %, but when the polypeptone was previously hydrolyzed with acid, the effect was diminished, indicating that its effect may be due to the small amount of vitamin B_1 or B_2 contained in it.

Yeast nucleic acid also shows some activity, viz. when 2–2.5 mg% is administered, no more pyruvic acid is accumulated. Purine and pyrimidine bases and their related substances such as uric acid, caffeine, adenine, guanine, alloxane, barbituric acid, uracil, cytosine, thymine, and orotic acid, were tested and none of them showed any activity. It is not yet clear whether the activity of yeast nucleic acid is attributable to its chemical constituents or to contamination from small amounts of some active substances.

*3. B_1-Homologues**:*— Following the clarification of the structure of vitamin B_1, many derivatives and homologues of it were synthesized. And whether they exist in nature or not, their physiological effects were being examined. Some of them were shown to have antiberiberi activity compared equal to or even stronger than vitamin B_1, but on the other hand, several homologues counteract the activity of vitamin B_1. The latter substances are called anti-B_1 or antithiamins.

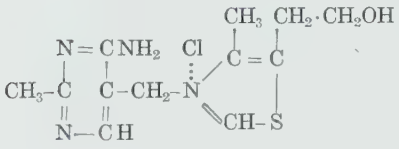
The present authors received some of these derivatives from Dr. T. Matsukawa, one of the investigators in this field, and carried out experiments to test their effects on the pyruvic acid accumulation of *M. mandshuricus*.

These substances can be classified physiologically as follows:

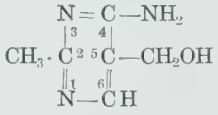
a) Substances, like vitamin B_1, depressing the accumulation of pyruvic acid upon being added in comparatively small amounts, and without inhibiting the normal metabolism of the microorgamism even at higher concentrations.

B_1-disulfide (B_1SSB_1) of Zima, and SB_1 of Matsukawa are representatives of this group. Their "antiberiberi" activities are about the same as vitamine B_1, and espacially the activity of the former cannot be differentiated

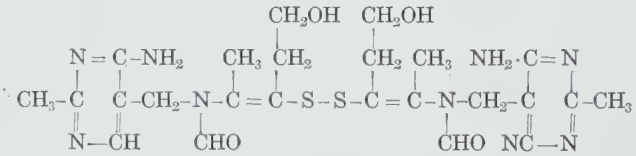
* Vitamin B_1 and B_1-Homologues



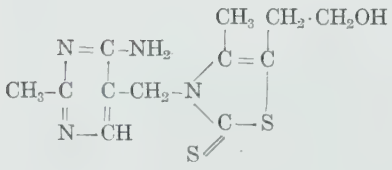
B_1



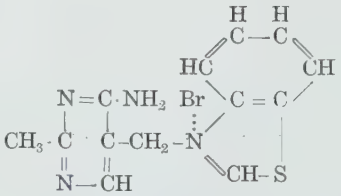
B_1-Pyrimidine



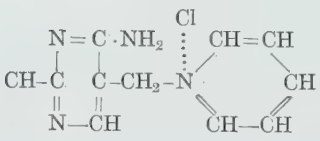
B_1SSB_1



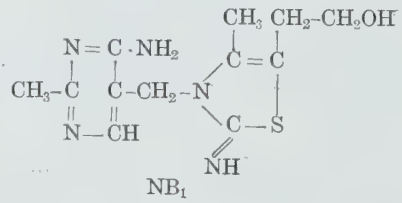
SB_1



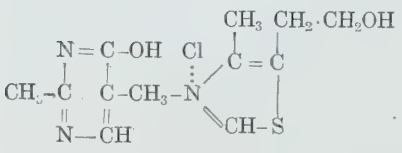
Benzo-B_1



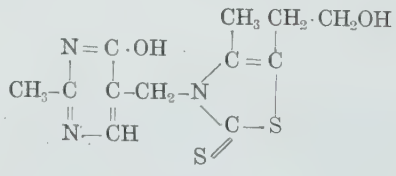
Pyridine-B_1



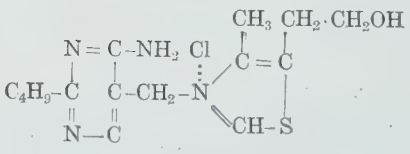
NB_1



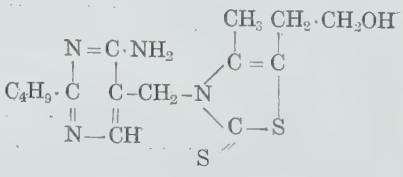
Oxy-B_1



Oxy-SB_1



Butyl-B_1



Butyl-SB_1

from B_1, while the latter substance is somewhat weaker (1/1.2) (Tables 6 and 7).

Table 6. Effect of B_1SSB_1

(Initial glucose 1,246 mg, 35 ml, 6 days)

B_1SSB_1 μg/35 ml	0	0.1	0.2	0.3	0.5	1 mg
Sugar consumed mg	562	819	723	766	675	721
Pyruvic acid 〃	57	55	34	21	trace	trace
Ethanol 〃	124	230	210	224	189	211
Dried pellicle 〃	20	20	20	20	20	20

Table 7. Effect of SB_1

(Initial glucose 1,379 mg, 35 ml, 5 days)

SB_1 μg/35 ml	0	0.1	0.3	0.5	1.0	5 mg
Sugar consumed mg	872	911	1,035	1,030	1,079	1,043
Pyruvic acid 〃	82	51	31	18	trace	trace
Ethanol 〃	210	230	279	299	270	236
Dried pellicle 〃	45	35	48	42	47	40

The substance ordinary called benzo-B_1 (benzothiazolo-B_1) can be classified into this group, but its activity is less than 1/1000 of vitamin B_1 (Table 8).

Table 8. Effect of benzo-B_1

(Initial glucose 1,365 mg, 35 ml, 6 days)

Benzo-B_1 μg/35 ml	0	5	10	25	50	5 mg
Sugar consumed mg	802	1,319	1,310	1,074	1,082	1,236
Pyruvic acid 〃	84	77	46	20	18	trace
Ethanol 〃	135	345	342	319	311	267
Dried pellicle 〃	60	85	100	60	70	60

Pyridine-B_1 and NB_1 are known as antivitamins for higher animals, but for *M. mandshuricus* they and B_1 act almost identically (Tables 9 and 10).

Table 9. Effect of pyridine-B_1

(Initial glucose, 1,225 mg; incubation 4 days)

Pyri-B_1 μg/35 ml	0	0.25	0.5	0.75	1000
Sugar consumed mg	700	945.4	1020.5	889.1	1041.4
Pyruvic acid 〃	125.8	61.2	39.4	8.0	2.8
Ethanol 〃	131.1	248.9	283.5	235.3	289.2
Dried pellicle 〃	60	42	60	50	55

Table 10. Effect of N-B_1
(Initial glucose, 1,113.0 mg; incubation, 6 days)

N-B_1 μg/35 ml	0	0.2	0.4	1	1,000
Sugar consumed mg	1,000.4	1,072.4	1,113.0	1,044.1	1,113.0
Pyruvic acid 〃	109.4	28.2	22.2	6.2	2.1
Ethanol 〃	200.9	277.6	292.3	296.6	310.7
Pellicle 〃	60	55	60	60	60

According to the authors' experiments, methyl and amino groups, attached respectivey at the 2 and 4 positions of the pyrimidine-nucleus, appear to be responsible for the antipyruvic activity.

b) Above a certain concentration, substances of this group depress the accumulation of pyruvic acid remarkably and also inhibit the general metabolism to some extent. Antivitamins such as oxy-B_1 and oxy-SB_1 are included in this group. We could not find their exact effect against antiberiberi activity of vitamin B_1 or SB_1, but the growth inhibiting action of oxy-B_1 was cancelled out by minute amounts of vitamin B_1 (Table 11).

Table 11. Antagonism between oxy-B_1 and B_1
(Incubation, 4 days)

Oxy-B_1 μg/35 ml	0	25	50	50	100	100	2,000	2,000
B_1 〃	0	0	0	1	0	1	0	1
Initial glucose mg	1,393	1,393	1,393	1,302	1,393	1,302	1,393	1,302
Sugar consumed 〃	671	682	608	1073	447	1008	175	645
Pyruvic acid 〃	65	69	34	trace	trace	trace	trace	trace
Ethanol 〃	167	178	134	276	81	268	26	161
Pellicle 〃	47	40	35	52	28	40	+	23

c) Only one substance has been found. Butyl-B_1 (methyl radical of pyrimidine nucleus of vitamin B_1 was substituted by butyl) is it that reveals to increase the accumulation of pyruvic acid, and this action is cancelled out by the presence of vitamin B_1, B_1-pyrimidine, pyridine-B_1, etc. (Table 12 (a), (b)).

Table 12 (a). Effect of butyl-B_1
(Initial glucose, 1,193.5 mg; incubation, 4 days)

Butyl-B_1 μg/35 ml	0	0.1	0.25	0.5	50
Sugar consumed mg	763.7	775.7	844.5	850.5	246.5
Pyruvic acid 〃	86.0	75.9	82.3	76.5	51.8
Ethanol 〃	167.9	168.5	169.6	198.4	40.8
Pellicle 〃	50	70	60	70	+

Table 12 (b). Antagonism between butyl-B_1 and B_1
(Initial glucose 1,148.0 mg; incubation 4 days)

Butyl-B_1 μg/35 ml	0	0	50	500	1,500
B_1 〃	0	1	1	1	1
Sugar consumed mg	580.5	909.0	670.2	385.5	315.5
Pyruvic acid 〃	105.5	10.2	52.8	155.0	93.8
Ethanol 〃	123.6	258.7	178.2	54.6	37.3
Pellicle 〃	50	70	45	20	10

Pyruvic acid stabilizing effect of butyl-B_1 can clearly be seen in the secondary culture of *M. mandshuricus*. The pellicle of the mould cannot produce any pyruvic acid in the secondary culture medium which contains only glucose, while respiration and alcohol production are carried out normally. But, if the pellicle is cultured in the presence of butyl-B_1, a noticeable amount of pyruvic acid is accumulated (Table 13).

Table 13. Effect of butyl-B_1 upon the secondary culture of the pellicle of *M. mandshuricus*
(Pellicle: 5 days old, initial glucose: 1,000 mg, incubation 44 hours)

Butyl-B_1 mg/30 ml	0	1
Sugar consumed mg	460.5	493.4
Pyruic acid 〃	**trace**	**44.3**
Ethanol 〃	80.5	80.2
Pellicle used 〃	120	125

As will be discussed later, this observation appears to be very important for elucidating the mechanism of pyruvic acid accumulation. But, since the effect by 150–200 μg of butyl-B_1 can be compensated by only 1 μg of vitamin B_1, the actual factor responsible for pyruvic acid accumulation may not be butyl-B_1 itself but some other far more effective substance.

Be that as it may, from the fact that the data obtained here with B_1SSB_1 and SB_1 conincide very well with the results of Zima and Ninomiya obtained by using rats and rice birds respectively, there will be some value in using *M. mandshuricus* preliminarily as to the test organism for anti-beriberi activities of B_1-homologues.

4. Metabolic poisons —For comparison, actual results obtained by oxy-B_1 and presented in Table 11 will be considered here again.

Almost no effect can be observed when the oxy-B_1 concentration is below 25 μg per 35 ml. At the concentration of 100 μg per 35 ml, not only does it completely arrest the pyruvic acid accumulation, but it also depresses the normal fermentation and reproduction of the mould considerably. If oxy-B_1 is increased to 2 mg per 35 ml, the inhibitory action upon the

normal metabolism becomes more distinct, but this inhibition can never be seen when only 1 μg of vitamin B_1 is present in the medium. In other words oxy-B_1 can be considered as an inhibitor of metabolism and its depressing action on pyruvic acid appears to have some relationship to the metabolic inhibition. Thus, the authors examined several well-known inhibitors from this aspect.

p-Chloromercuribenzoate and monoidoacetic acid: — These two substances act in the same way as oxy-B_1. Namely, they act as inbibitors against the general metabolism, but inhibit the pyruvic acid accumulation to a greater extent. The inhibitory activity of PCMB is about the same with oxy-B_1 (Table 14).

Table 14. Effect of inhibitors
(Initial glucose 1,319 mg, 35 ml)

Inhibitor	P.C.M.B	Mono-iodo-acetic acid	K_3AsO_3	NaF	Control
Quantity of inhibitor	100 μg	2 mg	10 mg	2 mg	
Sugar consumed mg	648	809	1,071	1,319	1,212
Pyruvic acid 〃	trace	trace	36	56	97
Ethanol 〃	157	142	285	319	290
Dried pellicle 〃	15	60	35	70	75
Period of inocubation. days	10	10	7	7	7

The inhibitory activity of monoiodoacetic acid is about one twentieth of oxy-B_1 or PCMB.

Barium ion: — In the course of studies on lactic acid production by *Rhizopus*-group, the authors found that the yield of lactic acid was markedly improved by barium ion. But in the case of pyruvic acid accumulation by *M. mandshuricus*, barium ion acts like previously listed inhibitors. It depresses carbohydrate metabolism and more seriously the pyruvic acid accumulation (Table 15).

Table 15. Effect of barium ion
(Initial glucose 1,211 mg, incubation 6 days)

Ba^{++} mg/35 ml	20	50	100	200
Sugar consumed mg	684	422	292	0
Pyruic acid 〃	92	trace	trace	0
Ethanol 〃	167	122	90	0
Pellicle 〃	30	+	+	0

It is noteworty that a single substance accelerates lactic acid fermentation on one hand, and retards pyruvic acid formation on the other hand. This reveals that the two fermentations take place by entirely different mechanisms.

In general, it may be concluded that the inhibitors not only depress the normal metabotisms of *M. mandshuricus*, but also the pyruvic acid accumulation of the organism to a greater extent.

Of course, the mechanisms of the pyruvic acid depressing effect of inhibitors might not be the same with vitamins. However, the results obtained henthforth can be summarized as follows.

Table 16. Substances which prevent pyruvic acid accumulation *M. mandschuricus*

	Concentration of the substance for		Inhibition against normal metabolism
	half depression	complete depression	
B_1	0.5 μg%	2 μg%	−
B_1-Pyrimidine	0.6 〃	4 〃	−
B_1SSB_1	0.6 〃	2 〃	−
SB_1	0.5 〃	3 〃	−
Benzo-B_1	30 〃	300 〃	−
Pyridine-B_1	0.7 〃	2 〃	−
NB_1	0.3 〃	3 〃	−
Oxy-B_1	150 〃	300 〃	+
Oxy-SB_1	3 mg%	4.5 mg%	+
B_2	18 μg%	40 μg%	−
Yeast nucleic acid	700 〃	2.5 mg%	−
"Polypeptone"	10 mg%		−
PCMB		300 μg%	+
Monojodoacetic acid		6 mg%	+
Ba··		100 mg%	+

Mechanism of Pyruvic Acid Accumulation
(A Consideration as to the Cause of Beriberi)

The inscription that "the syptom of beriberi appears when vitamin B_1 is deficient and the symptom is cured by administering the vitamin" has already become classical. Theme of the present day is to know why does the symptom follow the B_1-deficiency. According to the authors' conclusion, the direct cause of beriberi is abnormal accumulation of pyruvic acid. Now we want to consider by what mechanism pyruvic acid is accumulated, viz. symptom of beriberi does appear.

In the case of *M. mandshuricus*, the relationship between sugar consumption, alcohol production, and pyruvic acid accumulation is illustrated in Fig. 2. Scales are altered for the sake of convenience but we can see the three curves are parallel each other. Though the parallelism between the upper two curves may be regarded as reasonable, it is very curious to see that pyruvic acid, in spite of only being an intermediate substance, also runs parallel. If pyruvic acid really be an intermediate substance, it

must at least begin to decrease when the substrate sugar faded away.

If vitamin B_1 was present from the beginning of the fermentation, pyruvic acid could never appear as described repeatedly. But when the vitamin was added during the fermentation process, pyruvic acid accumulation was interrupted and the increment of alcohol production occurs at a slightly faster than in the control test, but the pyruvic acid which has already been formed does not show a significant tendency to decrease. Consequently, we cannot see any evidence for the strengthening of cocarboxylase by vitamin B_1. In other words, we can say that, though vitamin B_1 can arrest the accumulation of pyruvic acid, it cannot decompose the preformed pyruvic acid. Again, though it may be somewhat audacious, the author believe that it would not be a mistake to say "vitamin B_1 is a powerful agent in preventing beriberi, but is unable to cure the symptom".

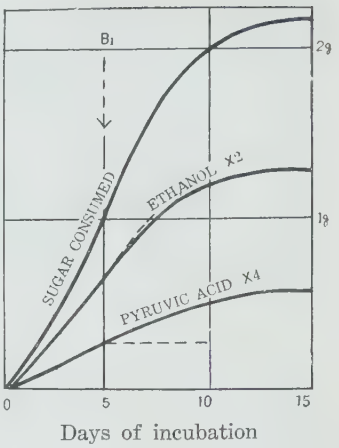


Fig. 2. Correlation between sugar-consumption, ethanol-production, and pyruvic acid-accumulation
(Dotted lines show when 2 $\mu g\%$ of B_1 was added during the fermentation process)

The results observed in microorganism cultured in a limited space may of course be not applicable to freely living man and animals. In man and animals abnormal substances formed in the tissuse are eventually discharged through the circulatory and excretory systems. So if the new accumulation of pyruvic acid is interrupted, preformed acid decreasses to the normal level and beriberi may be cured unless there exists any abnormality in these systems. Beriberi should be cured by the recovering mechanism of the patient with the aid of the preventive power of vitamin B_1.

It is very curious that pyruvic acid, if once accumulated, does not easily decrease even by supplying sufficient quantities of vitamin B_1, and the ethyl alcohol increases independently of the pyruvic acid.

In order to explain this phenomenon, the authors first imagined an alcoholic fermentation performed without passing through the stage of pyruvic acid, but such an idea does not agree with the general present day knowledge. We arrived at the conclusion that it might be most appropriate to explain the phenomenon, by assuming that pyruvic acid has two chemical structures, namely stable and labile.

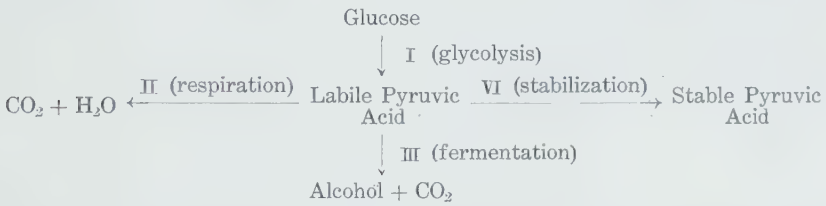
Ordinarily available pyruvic acid may be considered as a *keto*-form, but, since *M. mandshuricus* cannot decompose this form, the authors consider this as a stable form. On the other hand, the pyruvic acid produced intermedially via phosphoglyceric and phosphopyruvic acids must be a biochemically labile *enol*-form.

Here again the authors want to introduce an idea that there is a factor having enzymic nature called "pyruvic acid stabilizing factor" which stabilizes the *enol*-form into *keto*-form.

$$\underset{\text{Labile}(\textit{enol})\text{-form}}{CH_2{:}C(OH)\cdot COOH} \xrightarrow{\text{"Pyruvic acid stabilizing factor"}} \underset{\text{Stable}(\textit{keto})\text{-form}}{CH_3\cdot CO\cdot COOH}$$

If a part of *enol*-pyruvic acid were caught by this factor during alcoholic fermentation, the stabilized *keto*-form must be accumulated, because *M. mandshuricus* cannot utilize the latter form. Yeast, on the other hand, can freely decarboxylate both forms, and pyruvic acid could never be accumulated even if the organism had a strong stabilizing factor.

Path of sugar decomposition by *M. mandshuricus* can be written briefly as follows:



We have seen that there are several types of reactions which depress the accumulation of pyruvic acid. They can be conveniently understood if we imagine the upper scheme as a stream of water having several branches and flowing along the directions pointed out with arrows.

1) Vitamin B_1, B_1-pyrimidine, SB_1, B_1SSB_1, NB_1, pyridine-B_1, benzo-B_1.

As these substances favour the respiration and alcoholic fermentation by making smooth the flows of II and III, there is no excess left for the accumulation of pyruvic acid by reaction IV.

2) Vitamin B_2.

This substance makes smooth especially the oxidative decomposition. The main flow runs along II. Accordingly, not only reaction IV, but also the normal alcoholic fermentation III is relatively depressed.

3) Oxy-B_1, oxy-SB_1, and metabolic inhibitors.

So-called antivitamins such as oxy-B_1 and oxy-SB_1 inhibit, like metabolic inhibitors such as *p*-chloromercuribenzoate and monoiodoacetic acid, the entire metabolisms and above all reaction IV, the stabilizing of pyruvic acid. The mechanism of the inhibitory action is not yet clear, but the growth inhibiting action of the antivitamins can easily be prevented by minute amounts of vitamin B_1 or SB_1.

4) Butyl-B_1 and butyl-SB_1.

These antivitamins appear to be models of pyruvic acid stabilizing factor. Namely in the presence of these substances pyruvic acid tends to increase, while the growth of the mould is more or less inhibited. Pyruvic acid stabilizing effect of butyl-B_1 is antagonized by a quantity of about 1/150–200 of vitamin B_1.

The phenomenon of pyruvic acid accumulation — beriberi symptom — may reasonably be attributed to some factor being somewhat enzymatic be in nature. The factor was shown to exist actually in comparatively younger culture-solutions but fades away in the last stage of fermentation. The authors have not yet identified such a factor, but saw its model at butyl-B_1. Namely, in its presence, pyruvic acid accumulates in the secondary culture of *M. mandshuricus*, in which otherwise the acid is never detected at all (Table 13).

Although their purposes are somewhat different from ours, Utiger and Nord et al. published papers about the relationship between pyruuic acid and vitamin B_1 in *Phycomyces blakesleeanus* and *Fusarium lini* respectively.

However small they may be, since microorganisms are also living creatures, their resemblance to human beings should not be under-estimated.

Whether it is adequate or not to call it beriberi, we at least saw a symptom caused by B_1-dificiency which exists in microorganisms. It must be a delight to microbilogists if they can contribute to human pathology by using appropriate microorganisms.

Postscript

In this communication, data from the following papers (1~6) were considered from a particular point of view. The substance of the treatise (7) published in Japanese under the same title has been translated here by extending with new experimental results.

KITAHARA and FUKUI: On the Pyruvic Acid Fermentation.

(1) Selection of appropriate species from *Mucor*-group, and examination of conditions for pyruvic acid formation. *J. Fermentation Technol.* **29**, 287 (1951).

(2) Possibility of biochemical determination of vitamin B_1 by *Mucor mandshuricus*. *ibid*, **29**, 287 (1951).

(3) Substances which inhibit the accumulation of pyruvic acid. *ibid*, **29**, 326 (1951).

(4) A consideration of the mechanism of pyruvic acid accumulation by *M. mandshuricus*. *ibid*, **29**, 378 (1951).

(5) Three ways which depress the accumulation of pyruvic acid. *ibid*, **30**, 267 (1952).

(6) Effect of antithiamins upon the pyruvic acid accumulation. *ibid*, in preparation.

KITAHARA and FUKUI: (7) Beriberilike Symptoms in Microorganisms "A View on the Fermentation Industries" Osaka (1952).

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THE DECOMPOSITION OF L-(+)-TARTARATE BY THE MICROORGANISMS

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L-(+)-Tartaric acid is widely distributed among the higher plants, and many microorganisms are known to decompose it. In view of the significant role of the organic acids present in plants in the intermediary metabolism, it might be supposed that tartaric acid also plays some role in the metabolism of living cells, especially in plants or microorganisms. There have been several reports on the microbiological breakdown of this acid[14,19,21,24], but they were only qualitative or taxonomical studies and at present the metabolic behavior of this acid is scarcely known.

Therefore the present study was undertaken in order to clarify these problems. *Pseudomonas incognita* was used in this study. It was isolated from soil, and investigated with reference to aerobic and anaerobic decomposition of tartarate. Some results obtained will be reported in this paper.

EXPERIMENTAL METHODS

1. Bacterium used: Several strains which readily decompose tartarate were isolated from various sources by the enrichment technique[16] and one of them, isolated from the soil and identified as *Pseudomonas incognita*[16,17], was used in this study.

This strain, *Pseudomonas incognita strain 3L*, was usually grown in the tartarate-bouillon medium (NH_4-tartarate 1.2%, meat extract 0.8%, peptone 0.5%, NaCl 0.5%, pH=6.0) at 30° for 40 to 48 hrs. by stationary culture (anaerobically grown cell) or for 16 to 24 hrs. at 25°C by shaking culture (aerobically grown cell). After harvesting, the cells were washed with 0.2% KCl solution, resuspended in 0.2% KCl and used as resting cell suspensions. When dried cell preparations were used, they were prepared by vacuum-drying the washed dense cell suspension over $CaCl_2$.

2. Manometric and chemical methods: O_2 consumption and CO_2 evolution were followed in the conventional Warburg apparatus at 30°C with air as the gas phase for the aerobic experiment or with N_2 for the anaerobic experiment.

DL-Phosphoglycerate was prepared by Kiessling's method[9]. D-(−)-Phosphoglycerate prepared with yeast was kindly supplied by Mr. K. Omura. Phosphotartarate was prepared by the method of Neuberg et al. from the L-(+)-tartarate and phosphorus oxychloride[15].

Tartarate[13], inorganic phosphate[6], acetoin[26], and diacetyl[26] were determined colorimetrically. Succinate[23] and pyruvate[23] were determined manometrically. D-(−)-Malate was also determined manometrically by the decarboxylation method using the resting cell of malate adapted *Lact. arabinosus*.

Volatile acids were separated from the incubation medium by steam distillation. Formic acid was determined by the permanganate oxidation method. After the oxidation, the distillate was redistilled and acetic acid was determined by titration with alkali.

Paperchromatography of organic acids was performed by the method of Lugg et al.[11] and that of keto acids by the method of Cavallini et al.[5]

3. Measurements of radioactivity: Carbon dioxide evolved was absorbed in alkali, precipitated as $BaCO_3$, dried and counted by the conventional Geiger-Müller counter with an end window. The counted data was corrected for self absorbtion.

Formic acid was degraded by permanganate oxidation and counted as $BaCO_3$. Acetic acid was isolated as Na salt from the steam distillate after the removal of formic acid and counted as such.

Pure succinic acid was isolated from the reaction medium by ether extraction and decomposed by Van Slyke-Folch wet oxidation[4] and counted as $BaCO_3$. The specific activity of carboxyl carbon of succinate was obtained by degrading succinate to CO_2 and lactic acid by the method of Foster et al.[7] The specific activity of methylene carbon was calculated by the difference between total specific activity and that of carboxyl carbon and also confirmed by measuring directly the 2,4-dinitrophenylhydrazone of acetaldehyde which was obtained from the non-carboxyl carbons of lactic acid by $KMnO_4$ oxidation[8].

Tartaric acid was isolated as acid potassium salt and degraded by the Sprinson's periodate oxidation method[20] according to the following equation.

$$\begin{array}{l} \overset{2}{\mathrm{CH(OH)}}\text{–}\overset{1}{\mathrm{COOH}} \\ \quad\vert \\ \underset{3}{\mathrm{CH(OH)}}\text{–}\underset{4}{\mathrm{COOH}} \end{array} \xrightarrow{+3\mathrm{HIO_4}} \overset{2,3}{2\,\mathrm{HCOOH}} + 2\,\overset{1,4}{\mathrm{CO_2}} + \mathrm{H_2O} + 3\,\mathrm{HIO_3} \quad \text{.....(1)}$$

$$\overset{2,3}{\mathrm{HCOOH}} \xrightarrow[\text{in alkali}]{+\mathrm{KMnO_4}} \overset{2,3}{\mathrm{CO_2}} \quad \text{.....(2)}$$

Carbon dioxide, evolved by reaction (1) and (2) corresponding to the carboxyl and –CH(OH)– carbons of tartarate respectively, was counted as $BaCO_3$.

EXPERIMENTAL RESULTS

Part 1. Anaerobic decomposition of L-(+)-tartarate.

1. Identification of the decomposition products[16].

The decomposition products of tartarate were examined using the

resting cell suspension. The composition of the reaction mixture was as follows: Na-L-(+)-tartarate (8.365 g of free acid), $MgSO_4 \cdot 7H_2O$ 0.4 g, tap water containing 1.2 g dry weight of cells 100 ml., M/15 phosphate buffer (pH=7.0) 700 ml. Total volume 800 ml. After the incubation of 73 hrs. at 30°C the fermentation medium was analyzed. By the addition of 2,4-dinitrophenylhydrazine to the acidified medium a very small amount of hydrazone, which was insoluble in alkali, was obtained, but owing to its small quantity it's identification could not be achieved. The distillate of the neutral distillation was examined for alcohol, acetone, diacetyl, and acetaldehyde, but none of these compounds were found. Acetic acid was isolated by acid-steam distillation and identified as *p*-bromphenacyl ester (m.p.=82.5°, not depressed by mixing with a known sample of *p*-bromphenacyl ester) and also as the Ag salt (Calculated for Ag acetate, Ag 64.7%; Found 64.6%).

Formic acid was also detected by the calomel test in the acid-steam distillate, but its quantity was very small.

Succinic acid was isolated from the acidified medium by continuous ether extraction, recrystallized from water, and identified by the mixed melting point (m.p.=183°).

Besides succinic acid and tartaric acid no other organic acid was found when tested with paperchromatography. Thus the main fermentation products were confirmed to be acetic acid and succinic acid.

2. Balance study.

The balance study on the anaerobic decomposition of tartarate was performed using the resting cell suspensions. From the results shown in Table 1, it was clearly established that anaerobic decomposition of tartarate by this organism is performed according to the following equation.

$$3\,\begin{matrix}CH(OH)\text{–}COOH\\ |\\ CH(OH)\text{–}COOH\end{matrix} = 2\,CH_3COOH + \begin{matrix}CH_2\text{–}COOH\\ |\\ CH_2\text{–}COOH\end{matrix} + 4\,CO_2 + 2\,H_2O \quad \ldots\ldots(3)$$

Table 1. The anaerobic decomposition of L-(+)-tartarate.

Part 1.

	Observed value		Molar ratio
	mg	mM	
L-(+)-Tartaric acid consumed	397	2.63	3
Acetic acid formed	103.7	1.73	1.97
Succinic acid formed	94.0	0.80	0.91

Composition of the reaction mixture: Na-L-(+)-tartarate (915 mg as free acid), $MgSO_4 \cdot 7H_2O$ 50 mg, cell suspension (250 mg dry wt. of cells) 25 ml, M/15 phosphate buffer (pH=7.0) 75 ml. Total volume, 100 ml. Temp., 30°C. Time, 37 hrs. Anaerobic condition was maintained by evacuation.

Part 2.

	Observed value	Molar ratio
L-(+)-Tartaric acid consumed	2.00 μM	3
CO_2 evolved	2.58 μM	3.87

Evolved CO_2 was measured in Warburg's respirometer. Experimental conditions: Na-L-(+)-tartarate M/950 (2 μM), phosphate buffer M/28.5 (pH=5.6), cell weight 18.3 mg, total volume 1.9 ml. Temp., 30°C. Gas phase, N_2. Time, 50 min.

3. *Anaerobic decomposition of tartarate in the presence of radioactive bicarbonate*[17].

As shown in the previous section, the anaerobic decomposition of tartarate is performed according to equation (3). In order to clarify the mechanism of this reaction, anaerobic decomposition of this acid by the resting cells was conducted in the presence of radioactive bicarbonate and the incorporation of $C^{14}O_2$ into various compounds was examined. The large Thunberg tube was used for the reaction vessel, and the composition of the reaction medium is listed in Table 2.

Table 2. Composition of the medium for the anaerobic decomposition of tartarate.

Main compartment	Cell suspension* 20 ml. (285 mg dry wt.) M/15 phosphate buffer (pH=6.9) 15 ml.
Side arm	Na tartarate-$MgSO_4$ solution (tartarate 693 mg as free acid, $MgSO_4 \cdot 7H_2O$ 20 mg) 5 ml. $Na_2C^{14}O_3$ in N/10 NaOH 0.5 ml. (Ca, 1,400,000 c.p.m.)

* Cells obtained by stationary culture in the tartarate-bouillon medium for 40 hours at 30°C were used.

Incubation was done at 30°C for 3 hours under anaerobic conditions. The analysis of the reaction medium was carried out by the methods already described and the results obtained are listed in Table 3.

As shown in Table 3 very strong radioactivity was found in succinate and this radioactivity was found exclusively in carboxyl carbons. The specific activity of these carbons was 2.5 times as high as that of the final bicarbonate. Therefore this incorporation of C^{14} could not be explained by the mere exchange reaction between the succinic acid and CO_2. So either the reaction,

Table 3. Anaerobic decomposition of L-(+)-tartarate in the presence of radioactive bicarbonate.

	Chemical data		Isotopic data specific activity	Total activity
	mg	mM	c.p.m. per μM carbon	
Residual tartaric acid	134.7	0.898	1.85	6,645
{–CH–(OH)–			0	0
{–COOH			3.70	6,645
Tartaric acid consumed	59.0	0.393		
Acetic acid formed	14.9	0.247	0	0
Succinic acid formed	9.2	0.078	1565.6	488,000
{$-CH_2-$			0	0
{–COOH			3397.0	529,900
CO_2 formed	27.8	0.632	1358.5	858,600
Formic acid formed	1.8	0.040	860.6	34,400
Cell residue				7,610

Carbon recovery 94.2%.

$$\text{Tartarate} \longrightarrow C^*O_2 \longrightarrow \text{Succinate}$$

or, the reaction,

$$\text{Tartarate} \longrightarrow \underset{\uparrow\downarrow}{\overset{C^*O_2}{X^*}} \longrightarrow \text{Succinate}$$

must be postulated (These correspond respectively to Scheme I and II in the following discussion). This compound X* is the intermediate, well equilibrated with the bicarbonate in the medium, and considered to the oxalacetic acid. As expected, the organism was shown to have a high activity of oxalacetic acid decarboxylase both in the dried cell preparation ($Q_{CO_2}=26.6$ while Q_{CO_2} for tartarate is 8.4) and in the cell free extract (this extract was prepared by grinding the cells with alumina and extracting it with phosphate buffer (M/50, pH=7.0)). Therefore the primary fixation reaction of the isotope may be the Wood-Werkman reaction.

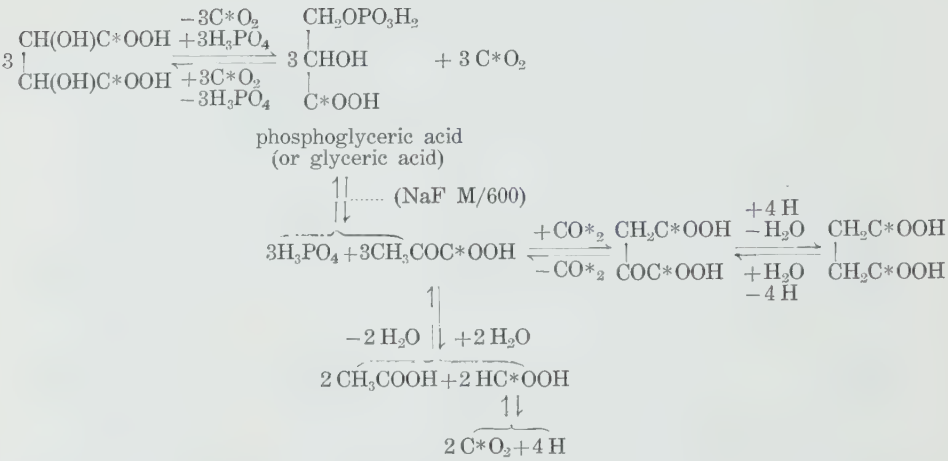
Besides, formic acid formed in small amounts was found to have about the same specific activity as that of the final bicarbonate, while acetic acid showed no radioactivity. This latter observation clearly excluded the Thunberg-Wieland reaction, i.e. the formation of succinate according to the following equation.

$$2\,CH_3COOH \underset{+2H}{\overset{-2H}{\rightleftarrows}} \begin{matrix} CH_2\text{–}COOH \\ | \\ CH_2\text{–}COOH \end{matrix}$$

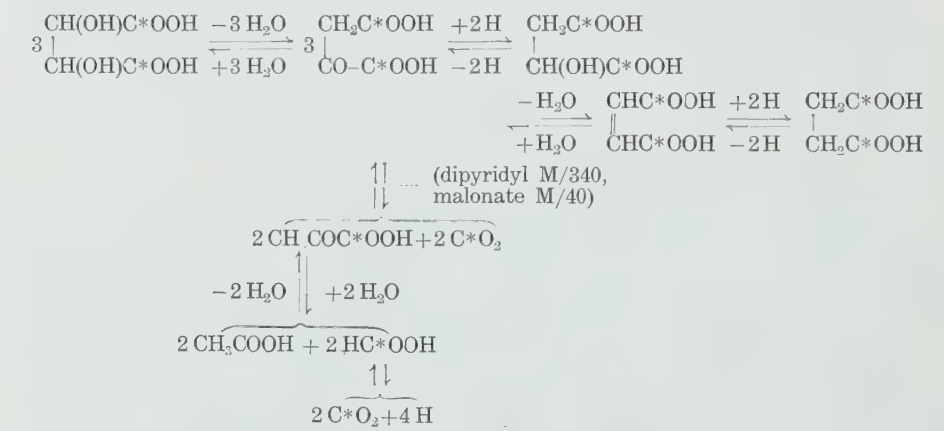
Residual tartaric acid showed weak activity and was labeled only in the carboxyl carbons.

These experimental results can well be explained either by Scheme I or II as follows:

Scheme I.



Scheme II.



It is supposed that phosphorylated compounds or coenzyme bound compounds are true intermediates, but these active forms were omitted for simplicity. C* = radioactive carbon.

Dotted lines represent the inhibition by the agents described (see text).

In the study of CO_2 fixation in green plants using the C^{14} isotope it is known that in a very short time phosphoglyceric acid contains most of the radioactive element[3]. From these results Calvin et al. proposed a C_4-dicarboxylic acid cycle[1] or its modified cycle[2] as a possible mechanism of the photosynthetic reaction. In these cycle the primary fixation reaction of CO_2 are $C_2+C_1=C_3$ (Phosphoglyceric acid) and $C_3+C_1=C_4$. In Scheme I, as shown above, the formation of phosphoglycerate by the decarboxylation of the tartarate was proposed as the first reaction of the decomposition

of tartarate, and since C^{14} incorporation into the carboxyl carbons of tartarate, as demonstrated experimentally, shows the reversibility of this reaction, tartarate seems to correspond to the "active C_4 compound" of the Calvin-Benson cycle. This consideration, together with the fact that tartaric acid is widely distributed in the plants, makes Scheme I very attractive. But the various experimental results excluded the possibility of Scheme I as the main path of the decomposition of tartarate and supported the occurence of Scheme II. These experimental approaches will be described in the following section.

4. *Examination of phosphoglycerate and oxalacetate as possible intermediates*[18].

a) Anaerobic decomposition of D-(−)-*phosphoglycerate,* DL-*phosphoglycerate, and* DL-*glycerate.*

Using resting cell suspension, we investigated anaerobic decomposition of D-(−)-phosphoglycerate, which was considered as a possible intermediate in Scheme I, and also of DL-phosphoglycerate and DL-glycerate manometrically either in the presence of tartarate or in its absence (Table 4).

The results presented in Table 4 show that DL-phosphoglycerate and DL-glycerate were hardly decomposed by this organism, and that DL-phosphoglycerate inhibited the decomposition of tartarate considerably and DL-glycerate a little. Whether these inhibitions are due to the presence of unnatural L form or not is unknown. On the other hand, D-(−) phosphoglycerate was decomposed at an appreciable rate, though this rate is still small compared to the rate of CO_2 production from tartarate, and no inhibition was observed in this case. Moreover, while DL phospho-

Table 4. Anaerobic decomposition of DL-phosphoglycerate, D-(−)-phosphoglycerate and DL-glycerate.

Exptl. No.	Time of incubation min.	Substrate added	CO_2 evolved μl	Inhibition %
1	50	Tartarate 4 μM	39.8	—
		DL-Phosphoglycerate 4.3 μM	0	—
		Tartarate 4 μM+ DL-Phosphoglycerate 4.3 μM	18.0	52.7
2	170	DL-Phosphoglycerate 8.1 μM	5.0	—
3	140	Tartarate 4 μM	81.5	—
		D-(−)-Phosphoglycerate 5 μM	20.1	—
		Tartarate 4 μM+ D-(−)-Phosphoglycerate 5 μM	103.9	0
4	95	Tartarate 10 μM	88.1	—
		DL-Glycerate 8 μM	3.4	—
		Tartarate 10 μM+ DL-Glycerate 8 μM	75.1	18.6

Cells used: Expt. 1 and 2, 6.3 mg dry weight, Expt. 3, 8.9 mg. Expt. 4, 4.2 mg.

glycerate was not decomposed by the dried cell preparation, D(−)-phosphoglycerate was decomposed at a rate similar to that of the resting cell.

The observed slow rate of metabolism of DL-glycerate might be due to the permeability effect of the cell membrane. However, in view of the fact that D-(−) phosphoglycerate, which may have more difficulty in penetrating the cell membrane than DL-glycerate, is metabolized at an appreciable rate, it seems likely that DL-glycerate must be phosphorylated to phosphoglycerate at the first step, and owing to the limited rate of this phosphorylation step, the slow rate of metabolism of this acid was observed. Therefore D-(−) phosphoglycerate was considered to be the most possible C_3 acid proposed in Scheme I, and the metabolic behavior of this acid was examined in the following experiments.

b) Quantitative studies on the decomposition of D-(−)-*phosphoglycerate.*

Scheme I assumes that tartarate is first decarboxylated to C_3 acid (possibly phosphoglyceric acid), and subsequently succinic acid is formed by the fixation of CO_2 into pyruvate derived from this C_3 acid.

Therefore, if in fact tartarate is decomposed according to Scheme I, the possibility of net CO_2 fixation yielding succinate may be great when phosphoglycerate is added as substrate and the following reaction sequence may be observed.

$$3 \text{ phosphoglycerate} = 3 \text{ pyruvate} + 3\,H_3PO_4 \quad \text{.....(4)}$$

$$2 \text{ pyruvate} + 2\,H_2O = 2 \text{ acetate} + 2\,CO_2 + 4\,H \quad \text{.....(5)}$$

$$\text{pyruvate} + CO_2 + 4\,H = \text{succinate} + H_2O \quad \text{.....(6)}$$

$$\text{Sum: } 3 \text{ phosphoglycerate} + H_2O = 2 \text{ acetate} + CO_2 + 3\,H_3PO_4 \quad \text{.....(7)}$$

According to equation (7), the formation of 1/3 molecule of CO_2 is expected per molecule of phosphoglycerate metabolized. But the experimental results (Table 5) showed that about 1 molecule of CO_2 was evolved per molecule of phosphoglycerate consumed. Since in this experiment the consumption of phosphoglycerate was determined by the measurement of the increase of inorganic phosphate, besides reaction (7) the following reaction due to phosphatase action

$$\text{phosphoglycerate} \longrightarrow \text{glycerate} + \text{phosphate}$$

might contribute to the increase of inorganic phosphate and therefore the

Table 5. The Decomposition of D-(−)-phosphoglycerate.

Exptl. No.	Phospho-glycerate added (μM)	Time of incubation	CO_2 evolved (μM)	Increase of inorg. P (μM)	$\frac{\mu M \Delta CO_2}{\mu M \Delta P}$
1	10	240	1.80	1.75	1.03
2	8	175	0.91	0.785	1.16

Each flask contained cells (6.9 mg dry weight in experiment 1 and 5.8 mg in experiment 2), M/150 phosphate buffer (pH=5.4) 0.5 ml. and water. Final volume; 1.8 ml. in experiment 1, and 2.0 ml. in experiment 2. temp.; 30°C. Gas phase; N_2.

true amount of CO_2 formation per molecule of phosphoglycerate metabolized may be greater than the figures shown in the last column of Table 5.

Therefore, it is clear that the CO_2 fixation reaction according to equation (7) was not the main reaction. Of course the intermediate compound does not necessarily follow the same metabolic course as the parent substance, but in this case, from the thermodynamic point of view[18], the CO_2 fixation reaction according to Scheme I may be performed more easily when phosphoglycerate rather than tartarate is the substrate. Therefore this experimental result makes Scheme I unlikely.

c) The effect of NaF on the anaerobic decomposition of tartarate and D-(−)-*phosphoglycerate.*

It is well known that enolase which catalyzes reaction (8) is easily inhibited by NaF and consequently phosphoglycerate accumulates in a culture of bacteria as well as in yeast and animal cells.

$$\text{D-(−)-phosphoglycerate} = \text{phosphopyruvate} + H_2O \quad \text{.....(8)}$$

If the decomposition of phosphoglycerate by *Ps. incognita* is also catalyzed by enzyme enolase, this decomposition will be inhibited by NaF. As expected, in the presence of M/600 NaF, both CO_2 production and inorganic phosphate release from phosphoglycerate were completely inhibited (Table 6). Therefore, if the decomposition of tartarate follows Scheme I which presupposed phosphoglycerate as an intermediate, the decomposition of tartarate should also be inhibited by NaF. But as shown in Table 6, no inhibition was observed in the presence of the same concentration of NaF and no phosphoglycerate was accumulated even when the tartarate decomposition was partially inhibited by the higher concentration of NaF. Therefore phosphoglycerate was excluded as a possible intermediate.

Table 6. Inhibition of the anaerobic decomposition of tartarate and D-(−)-phosphoglycerate by NaF.

Exptl. No.	Time of incubation (min.)	Substrate	NaF (final concn.)	CO_2 evolved (μl)	Inhibition (%)	Increase in inorg. *P* (γ)
1	130	Tartarate	—	65.5	—	+ 1.8
		Tartarate	M/150	13.8	79.0	+ 0.5
		Tartarate	M/300	29.5	54.9	+ 0.3
2	60	Tartarate	—	43.9	—	−*
		Tartarate	M/600	43.1	2.0	+ 1.5
3	175	Phosphoglycerate	—	20.3	—	+23.5
		Phosphoglycerate	M/300	0	100.0	+ 0.5
		Phosphoglycerate	M/600	0	100.0	+ 1.5

Experiments were carried out in Warburg's vessels. Each flask contained 5.8 mg (dry wt.) of cells, 20 μM of tartarate or 8 μM of D-(−)-phosphoglycerate as substrate, NaF as shown in the table, 0.5 ml. of M/150 phosphate buffer (pH=5.4) and water to make the final volume 2.0 ml. Temp.; 30°C. Gas phase; N_2.

* Not determined by accident.

d) Inhibition experiments with malonate, α,α'-dipyridyl and KCN.

From the results of the previous experiments the possibility of the occurence of Scheme I was excluded; consequently, the validity of Scheme II was examined next. Scheme II assumes oxalacetate as the first intermediate. If this is so, then the decomposition of tartarate should also be inhibited, when the decarboxylation of the oxalacetate is inhibited by suitable inhibitors. Although the specific inhibitor of the decarboxylation of oxalacetate was not known, malonic acid, in its higher concentration, is reported to inhibit the oxalacetate decarboxylase in addition to the succinic acid dehydrogenase[10,25], and α,α'-dipyridyl is reported to inhibit the decarboxylation of oxalacetate by *Bact. succinicum*[22]. Therefore the effects of these substances on the anaerobic decomposition of tartarate and of oxalacetate were investigated (Table 7).

Table 7. The effects of various inhibitors on the anaerobic decomposition of tartarate and oxalacetate.

Exptl. No.	Time of incubation (min.)	Substrate (final concn.)	Inhibitor (final concn.)	CO_2 evolved (μl)	Inhibition (%)
1	40	Tartarate (M/100)	—	56.7	—
			Malonate (M/200)	46.3	18.4
			Malonate (M/40)	31.0	45.4
2	30	Oxalacetate (M/210)	—	52.3	—
			Malonate (M/42)	25.3	51.6
3	40	Tartarate (M/85)	—	55.3	—
			Dipyridyl (M/1700)	42.7	22.8
			Dipyridyl (M/340)	30.0	45.7
4	24	Oxalacetate (M/240)	—	33.5	—
			Dipyridyl (M/330)	12.6	62.3
5	30	Tartarate (M/100)	—	97.8	—
			KCN (M/400)	34.8	64.4
			KCN (M/10)	0	100.0
6	30	Tartarate (M/450)	—	43.3	—
			KCN (M/1800)	45.8	0

Experiment 2 and 4 were performed with dried preparations. Boiled preparations were used to correct the non-enzymatic decomposition of oxalacetate. Other experiments were performed with resting cell suspensions.

As shown in Table 7 both reactions were inhibited by dipyridyl (M/340) and malonate (M/40) almost to the same degree. This result supported the occurence of Scheme II.

Table 7 also shows that the anaerobic decomposition of tartarate was inhibited by the higher concentration of KCN, a known keton reagent, but not by its lower concentration.

e) Anaerobic decomposition of D-(−)-*malate and pyruvate.*

The anaerobic decomposition of D-(−)-malate and pyruvate, the inter-

mediates presumed in Scheme II, were investigated. If the anaerobic decomposition of malate is performed according to Scheme II as in the case of tartarate, dismutation to succinate, acetate, and CO_2 according to equation (9) is to be expected.

$$3\,\begin{matrix}CH_2\text{–}COOH\\ CH(OH)\text{–}COOH\end{matrix} = 2\,\begin{matrix}CH_2\text{–}COOH\\ CH_2\text{–}COOH\end{matrix} + CH_3COOH + 2CO_2 + H_2O \quad \ldots\ldots(9)$$

The experimental analysis confirmed this supposition (Table 8).

Table 8. Anaerobic decomposition of D-(−)-malate.

	Observed value			Theoretical value molar ratio according to eq. (9)
	mg	μM	Molar ratio	
D-(−)-Malic acid added	42.9	320		
D-(−)-Malic acid remained	0	0		
D-(−)-Malic acid consumed	42.9	320	3.00	3
CO_2 formed	8.95	203.5	1.90	2
Succinic acid formed	20.1	170.2	1.59	2
Acetic acid formed	3.88	64.6	0.60	1

Experiments were carried out in a large Thunberg tube. Cells 40 mg dry weight, M/15 phosphate buffer (pH=5.4) 12 ml. Na-DL-malate 640 μM, and water. Total volume; 40 ml. Air was removed by evacuation. Temp.; 30°C. Time; 4 hrs.

Table 9. Anaerobic decomposition of pyruvate.

Exptl. No.		Observed value			Theoretical value molar ratio according to eq. (7)
		mg	μM	Molar ratio	
1*	Pyruvic acid added	25.4	288		
	Pyruvic acid remained	0	0		
	Pyruvic acid consumed	25.4	288	1.00	1
	Acetic acid formed	15.6	260	0.90	1
	Formic acid formed	2.1	46	0.16	
	Succinic acid formed	0.50	4.2		
	Acetoin formed	0.54	8.1		
	Diacetyl formed	0	0		
2**	Pyruvic acid consumed		3.6	1.00	1
	CO_2 formed		3.7	1.03	1
	H_2 formed		2.8	0.78	1

* Experimental conditions were the same as in Table 8 except that 288 μM of pyruvate was added instead of malate, and the time of incubation was 130 min.

** Determined manometrically in a separate experiments under the similar conditions.

It was already shown (Table 5) that net fixation of CO_2, as claimed in Scheme I, was not observed when phosphoglycerate was the substrate. In the next experiment further examination of the possibility of net CO_2 fixation reaction, $C_3+C_1 \rightarrow C_4$, was undertaken quantitatively using pyruvate as the substrate (Table 9). Table 9 shows that pyruvate is metabolized according to equation (10) and the formation of C_4 compounds such as succinate, acetoin, and diacetyl, was negligibly small.

$$CH_3COCOOH + H_2O = CH_3COOH + CO_2 + H_2 \quad \text{.....(10)}$$

In this case too then, the net CO_2 fixation reaction seems not to occur.

Part 2. Aerobic decomposition of L (+)-tartarate.

1. Oxidation of tartarate by resting cells.

The resting cell suspension of *Ps. incognita strain 3L* which was grown in a medium containing tartarate rapidly oxidizes tartarate without an appreciable lag (Non-adapted cells oxidize tartarate only after a 50 to 80 min. lag period.). The amounts of O_2 uptake and CO_2 output were measured manometrically. About 1.3 molecule of O_2 uptake and 2.6 molecule CO_2 output were observed per molecule of tartarate added. The observed RQ is about 2.0 at the end of the reaction.

Fig. 1 shows one example of such experiments.

Since these observed values are far below those required for the complete oxidation of tartarate (2.5 molecule O_2 uptake and 4 molecule CO_2 output per molecule of tartarate; RQ=1.6), the search for the oxidation products was attempted using the aerobically grown resting cell suspension. The composition of the reaction mixture was as follows: cell suspension (2.34 g (dry wt.) of cells in 0.2% KCl) 384 ml., M 15 phosphate buffer (pH=5.4) 576 ml., M/10 Na tartarate solution (containing 2.88 g free tartaric acid), total volume 1152 ml. This reaction mixture was divided into 20 flasks and was shaken at 25°C. After 250 min., when oxidation had ceased as judged manometrically in the parallel experiments, the reaction was arrested by acidifying the medium to pH 2 with 10 N

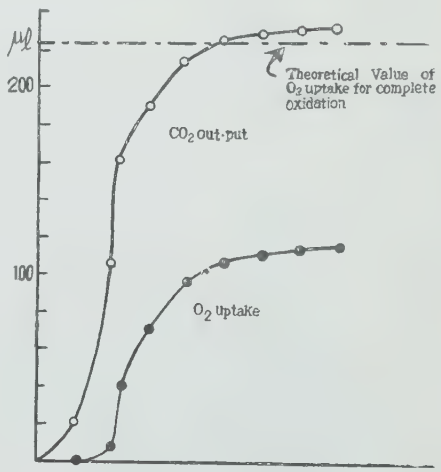


Fig. 1. The oxidation of tartarate by *Ps. incognita strain 3L.*

Composition of the reaction mixture: cell 5.1 mg dry wt. Na tartrate 4 μM, M/15 phosphate buffer (pH=5.4) 1.0 ml., total volume 1.7 ml. Temp., 30°C, Gas phase, air.

H_2SO_4 and the reaction medium was analyzed by methods similar to those described in experiment 1 of part 1. Very small amounts of pyruvate, unknown keto acid (possibly α-ketoglutarate judging from the Rf value of paperchromatography), and residual tartarate were detected but no other products were found.

2. The effect of 2,4-dinitrophenol on the oxidiation of tartarate.

As described in the previous section no accumulation of products was observed when tartarate was oxidized and yet the amount of O_2 uptake or CO_2 out put never reached the theoretical value of the complete oxidation. Therefore it is thought that a part of the tartarate may be assimilated and the other part may be completely oxidized. In order to test this assumption, the oxidation of tartarate was performed in the presence of 2,4-dinitrophenol, a known inhibitor of oxidative assimilation.

As shown in Table 10, the observed values for O_2 uptake and CO_2 output in the presence of M/20,000 2,4-dinitrophenol were close to the theoretical value for the complete oxidation of tartarate. Therefore, it seems likely that aerobically a part of tartarate is assimilated, while the other part is completely oxidized, and this oxidative assimilation is inhibited by 2,4 dinitrophenol.

Table 10. The oxidation of tartarate in the presence of 2,4-dinitrophenol.

	Observed value		Theoretical value for complete oxidation (Molar ratio)
	μM	Molar ratio	
L-(+)-Tartarate added	4.00	1.00	1.00
O_2 taken up	8.82	2.21	2.50
CO_2 evolved	13.5	3.38	4.00
RQ		1.53	1.60

Gas volume changes were measured in the Warburg's respirometer. Composition of the reaction mixture; Na-L-(+)-tartarate M/500, phosphate buffer (pH=5.4) M/30, 2,4-dinitrophenol 5×10^{-5} M, cells 3.2 mg dry wt., total 2.0 ml. Temp. 30°C. Gas phase, air. Time 93 min.

In the course of these experiments it was observed that 2,4-dinitrophenol (5×10^{-5} M) inhibited the early stage of the oxidation of tartarate but after 15 to 25 min. the oxidation proceeded at about the normal rate and eventually the amount of O_2 taken up or CO_2 produced exceeded that of the control reaction (Fig. 2).

Apparently 2,4-dinitrophenol prolonged the lag period which was observed for a short time in the normal oxidation by the adapted cell. Therefore it may be that the oxidation of tartarate requires activation by some sort of mechanism using phosphate bond energy and this step is inhibited by the 2,4-dinitrophenol.

3. The oxidation of various organic acids by the tartarate adapted and non-adapted cells.

Cells were grown in the absence of tartarate, harvested, and washed. Tartarate adapted and non-adapted resting cells were then prepared and examined as to their ability to oxidize various organic acids (Fig. 3 and 4).

No marked change following the adaptation to tartarate was observed except that the ability to oxidize acetate was depressed as a result of the adaptation to tartarate (Fig. 3; Qo_2 for acetate is 17.1 for non-adapted cell and 9.5 for adapted cell). D-(−)-phosphoglycerate and DL-glycerate were oxidized slowly both by the adapted and non-adapted cells and no marked difference between these two cases was observed (Fig. 4).

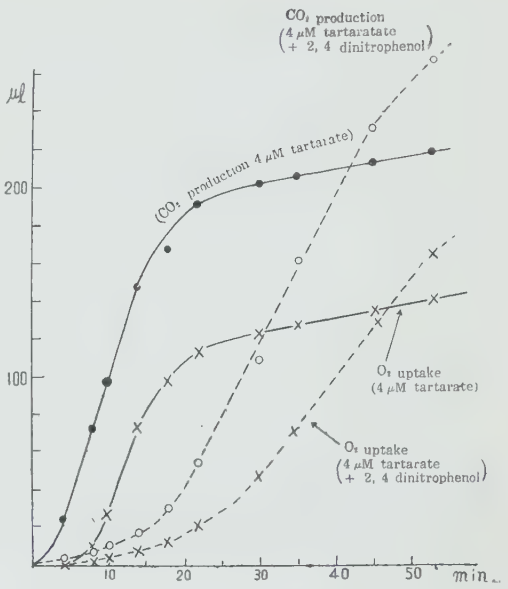


Fig. 2. The effect of 2,4-dinitrophenol on the oxidation of tartarate.

The composition of the reaction mixture was the same as that in Table 10 except that the cells used were 3.6 mg dry wt. per cup.

4. The oxidation of various organic acids by the resting cell grown in bouillon-tartarate medium.

The oxidation of various organic acids which might be the possible intermediates of the metabolism of tartarate were investigated using the cells grown in the usual bouillon-tartarate medium. Glycolic and fumarylglycidic acids were not oxidized. Dioxymaleic, DL-glyceric and DL-phosphoglyceric acids were oxidized very slowly. D-(−)-Phosphoglyceric acid was oxidized slowly (Qo_2=2–10 while Qo_2 for tartarate=70–220). On the other hand oxalacetic and pyruvic acids were oxidized at nearly same rate as tartarate without any appreciable lag period (See also Fig. 3 (a)).

The lag period of O_2 uptake in the oxidation of tartarate was more marked when the concentration of tartarate was decreased, while the evolution of CO_2 from tartarate was observed to proceed without any appreciable lag period (Fig. 1 and 2). Consequently the RQ at the early stage is far greater than 2 and, as the reaction proceeds, decreases gradually to 2, the value at the end of the reaction. In the experiment shown in Fig. 1, CO_2 output from 4 μM of tartarate after 10 min. was 107.4 μl or 4.7 μM while O_2 uptake was only 11.2 μl or 0.5 μM. Therefore, 1 molecule

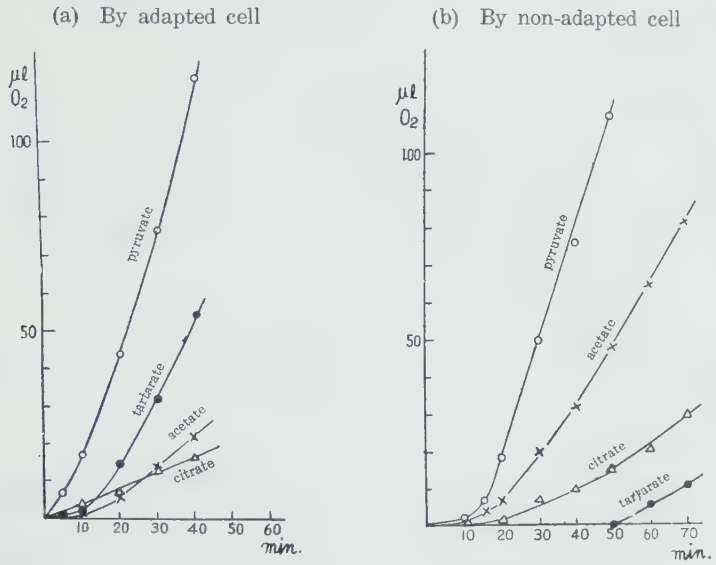


Fig. 3. The oxidation of various substrates by *Ps. incognita strain 3L.*

Cells were grown in a glucose medium (glucose 2%, $(NH_4)_2SO_4$ 0.2%, peptone 0.1%, K_2HPO_4 0.1% pH=6.8) at 30°C for 22 hrs., harvested, washed and the resting cell suspension was divided into 2 parts and shaken in M/53 phosphate buffer (pH=7.0) with and without tartarate (M/60). The two resulting cell suspensions, tartarate adapted and non-adapted cells, were harvested, washed and used for the experiment. Composition of the reaction mixture: cells 5.3 mg in (a) and 5.6 mg in (b), phosphate M/25 (pH=7.0), substrate as shown in the figure M/85, total volume 1.7 ml. Temp., 30°C. Gas phase, air.

of net CO_2 production from 1 molecule of tartarate was observed for this period.

These observations trongly indicate that the first step of the aerobic decomposition of tartarate is its decarboxylation via oxalacetate to pyruvate (eq. (11)) and not its oxidation to dioxymaleate before decarboxylation (eq. (12)).

$$\begin{array}{l} CH(OH)\text{–}COOH \\ | \\ CH(OH)\text{–}COOH \end{array} \xrightarrow{-H_2O} \begin{array}{l} CH_2\text{–}COOH \\ | \\ CO\text{–}COOH \end{array} \xrightarrow{-CO_2} CH_3CO\ COOH \longrightarrow CO_2 + H_2O \quad \ldots\ldots\ldots\ldots (11)$$

$$\begin{array}{l} CH(OH)\text{–}COOH \\ | \\ CH(OH)\text{–}COOH \end{array} \xrightarrow{-2H} \begin{array}{l} C(OH)\text{–}COOH \\ | \\ C(OH)\text{–}COOH \end{array} \xrightarrow{-CO_2} \begin{array}{l} CH_2(OH) \\ | \\ CO \\ | \\ COOH \end{array} \rightarrow CO_2 + H_2O \quad \ldots (12)$$

dioxymaleic acid

hydroxypyruvic acid

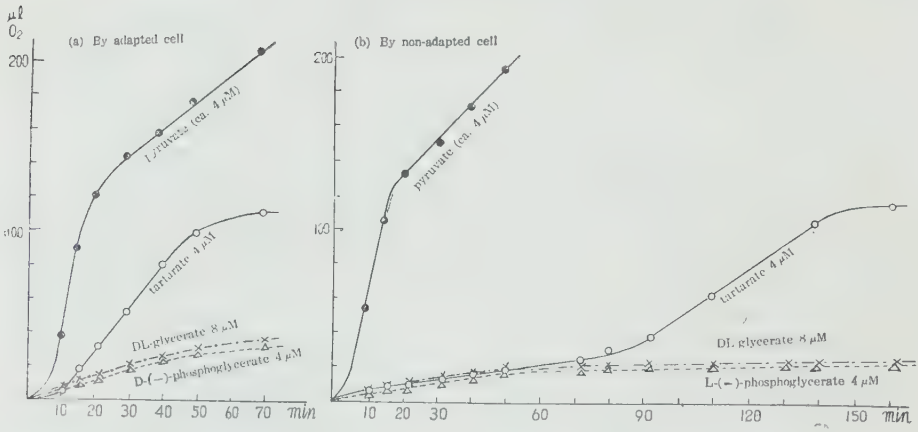


Fig. 4. The oxidation of various substrates by *Ps. incognita strain 3L.*

Cells were grown in an acetate medium (Na acetate·$3H_2O$ 1.2%, $(NH_4)_2SO_4$ 0.2%, K_2HPO_4 0.1%, pepton 0.1%, pH=6.4) at 25°C for 46 hrs. Tartarate adapted and non-adapted cells were prepared by a method similar to the one described in Fig. 4. Composition of the reaction mixture: cells 6.7 mg, phosphate M/31.5 (pH=5.4), substrate as indicated in the figure. Total volume, 2.1 ml. Temp., 30°C. Gas phase, air.

5. Search for the primary decarboxylation product.

When tartarate was decomposed aerobically CO_2 production was observed immediately while O_2 uptake started only after the lag period. Consequently it was supposed that the primary decarboxylation products might accumulate in the medium at the early stage. Therefore the search for this compound was attempted. The reaction mixture (M/36 phosphate buffer containing 4.8 g of tartaric acid and 1.52 g dry wt. of cell, pH=5.4, total volume 960 ml.) was shaken at 30°C for 10 min.

The reaction was then immediately arrested by adding H_2SO_4 and the medium was analyzed. Very small amounts of pyruvate and α-ketoglutarate were detected by paperchromatography (Rf=0.65 and 0.11 respectively). Control experiments did not show these keto acids. But their amounts were too small to account for the observed gas changes. Besides these keto acids and residual tartaric acid, no accumulation of organic acids was observed in the medium.

Therefore this attempt was fruitless.

6. The effect of arsenite and monojodacetate on the aerobic oxidation of tartarate and pyruvate.

If the first step of the aerobic decomposition of tartarate is its decarboxylation to pyruvate prior to its dehydrogenation, the O_2 uptake due to tartarate should be inhibited by the inhibitors which inhibit the oxidation of pyruvate. Morover, if this inhibitor is suitably selected the reaction from tartarate to pyruvate could be demonstrated without any confusion

by the oxygen uptake of the subsequent reaction.

Arsenite and monojodacetate were tested from this point of view (Table 11).

Table 11. The effect of arsenite and monojodacetate on the aerobic decomposition of tartarate and pyruvate.

Exptl. No.	Time of incubation (min.)	Substrate (final concn.)	Inhibitor (final concn.)	O_2 uptake		CO_2 out put		RQ
				Observed μl	Inhibition %	Observed μl	Inhibition %	
1	45	Tartarate (M/390)	—	91.0	—	216.0	—	2.37
			As_2O_3 (M/1200)	33.8	63	109.1	49	3.23
2	21	Pyruvate (M/780)	—	54.0	—	*		
			As_2O_3 (M/1200)	14.0	74	*		
3	30	Tartarate (M/525)	—	185.9	—	*	—	—
			$CH_2ICOONa$ (M/525)	6.3	97	41.3		6.1
4	20	Pyruvate (M/525)	—	106.9	—	*		
			$CH_2ICOONa$ (M/525)	14.1	86	*		

Cells used: 8.5 mg dried cells in Expt. 1 and 2. 5.0 mg intact cells in Expt. 3 and 4.

* Not determined.

As shown in Table 11 both arsenite and monojodacetate which inhibited the oxidation of pyruvate inhibited the oxidation of tartarate almost to the same degree. Particulary interesting are the results obtained with monojodacetate. This compound inhibited the O_2 uptake due to tartarate almost completely while CO_2 production still proceeded, though at very slow rate. Consequently, the RQ value is, after 30 min., still 6.1 in the presence of monojodacetate instead of 2.0 to 2.4 as in the normal case. These results support the above conclusion that the first step of the aerobic decomposition of tartarate is its decarboxylation to the pyruvate stage and not its dehydrogenation to the dioxymaleic acid stage.

Since DL-glycerate and phosphoglycerate are oxidized only at very slow rates and this rate is not increased by the adaptation to tartarate (Fig. 4) these C_3 conpounds are unlikely to be the intermediates in the decomposition of tartarate. Furthermore, in the anaerobic decomposition of tartarate, tartarate is decomposed according to Scheme II rather than Scheme I as already described. Therefore, aerobically too, tartarate may first be converted to pyruvate via oxalacetate and then oxidized by the usual TCA-cycle or a similar cycle (The appearence of α-ketoglutarate in addition to pyruvate at the early stage of tartarate oxidation suggests the occurrence of the TCA-cycle).

Part 3. Attempts to extract the enzyme system in a cell free state.

Thus far the study was performed with the intact cells or the dried

cell preparations. In order to prove more conclusively the first step of the decomposition of tartarate attempts were made to extract the enzyme system in a cell free state by means of various methods.

The dried cell preparations were ground in a ball mill and the freshly harvested cells were ground with alumina or sand. But in the clear supernatant solution obtained after high speed centrifugation no activity was ever found. On the other hand, in the particulate fraction the enzymatic activity was found occasionaly.

From the results of the 2,4-dinitrophenol experiments already described it was thought that the lack of the enzymatic activity in the supernatant fraction toward tartarate might be due to the lack of the activating mechanism of the phosphate bond energy. This possibility was examined. First the supernatant fraction was coupled with the plant mitochondria obtained from the mug bean according to the method of Maruo et al.[12] but without success. The addition of ATP to the supernatant also did not show any marked effect. However, when monophosphotartarate prepared according to the method of Neuberg et al.[15] was tested as the substrate, this compound was decomposed anaerobically by the enzyme preparation which did not show any activity upon tartarate and 1 molecule of CO_2 was evolved per molecule of phosphotartarate metabolized (as judged by the increase of inorganic phosphate) (Table 12). This result does not necessarily prove phosphotartarate to be the intermediate in the anaerobic decomposition of tartarate, but it strongly suggests the existence of some

Table 12. The anaerobic decomposition of phosphotartarate by the enzyme preparation.

Substrate added	CO_2 evolved		Inorganic P. increased	
	μl	μM	γ	μM
Phosphotartarate (4 μM)	26.1	1.17	37.7	1.22
Tartarate (4 μM)	4.8	0.21	3.7	0.12
Phosphotartarate (4 μM) +tartarate (4 μM)	28.7	1.28	39.0	1.26

Freshly harvested cells were chilled and ground with alumina and then extracted with 0.2% KCl solution. Alumina was removed by low speed centrifugation for 5 min. Then the cell debris was removed by high speed centrifugation (about 8,000 to 10,000 r.p.m.) for 10 min. Resultant cloudy, reddish yellow solution which contained some particulate matter was used as the enzyme solution. The reaction was carried out in Warburg's respirometer. The composition of the reaction mixture: enzyme solution 0.5 ml., M/150 phosphate buffer (pH=5.4) 0.4 ml., tartarate or phosphotartarate as indicated in the table. Total volume, 1.3 ml. Gas phase, N_2. Temp., 30°C. After 70 min. the reaction was stopped by adding 0.2 ml. of 10 N H_2SO_4. The precipitated protein was removed and inorg. P was determined in the supernatant solution.

mechanims which activates tartarate.

Phosphotartarate was also oxidized slowly by intact cells and by dried cell preparations (Qo_2=2.7 with the intact cells and 8.2 with the dried cell preparations).

Part 4. Possible mechanism of the formation of tartarate in the plant.

From the experiments described in this paper it was concluded that tartarate is metabolized via oxalacetate under aerobic as well as anaerobic conditions. Oxalacetate is situated at the key position of the tricarboxylic acid cycle or C_4-dicarboxylic acid cycle and at this point the metabolism of tartarate seems to be linkded with the main path of cellular metabolism. Since in the isotopic experiment shown in Table 2 and 3, the incorporation of C^{14} into carboxyl carbons of residual tartarate was observed, the reaction from tartarate to oxalacetate seems to be reversible and the formation of tartarate by this reaction may also be possible. In the plant tartarate is widely distributed, particularly in the leaf, and there the interconversion between this acid and other C_4 or C_6 acids is occasionaly observed. Though no direct experimental observations on the metabolism of tartarate in the plant have been made, it may be supposed from the results obtained in this paper that in plants too, tartarate may be formed or metabolized via oxalacetate and thus playing some role together with other stored organic acids. But the final conclusion on this point will require the direct demonstration of this reaction by an enzyme preparation obtained from plant tissue.

SUMMARY

Using *Pseudomonas incognita strain 3L* aerobic and anaerobic decomposition of L-(+)-tartarate was investigated.

Succinic and acetic acids were isolated and identified as the anaerobic decomposition products of tartarate by resting cells. Furthermore, from the balance studies it was clearly established that anaerobic decomposition of tartarate is performed according to eq. (3).

In order to clarify the mechanism of this reaction, anaerobic decomposition of tartarate by resting cells was conducted in the presence of radioactive bicarbonate and the incorporation of $C^{14}O_2$ into various compounds was examined. From the experimental results two schemes (Scheme I and II) were proposed to explain the observed incorporations of the isotope. But the following experimental results excluded the possibility of Scheme I as the main path of the anaerobic decomposition of tartarate by this organism.

(1) D-(−)-Phosphoglycerate was decomposed anaerobically and about 1 molecule of CO_2 was evolved per molecule of phosphoglycerate consumed, while 1/3 molecule of CO_2 should be expected if this compound were metabolized according to Scheme I. Therefore CO_2 fixation reaction according to Scheme I did not occur under these conditions. From the thermodynamic

point of view, the CO_2 fixation reaction according to Scheme I may be performed more easily when phosphoglycerate rather than tartarate is the substrate. Therefore this experimental result makes Scheme I unlikely.

(2) Similarly succinate formation by the CO_2 fixation reaction was not observed in the anaerobic decomposition of pyruvate.

(3) NaF, in the final concentration of M/600, completely inhibited the decomposition of phosphoglycerate, whereas the decomposition of tartarate was not inhibited in the same concentration. Therefore phoshoglycerate was excluded as the intermediate.

(4) Anaerobically, DL-glcerate was not metabolized by this organism and is not likely to be the intermediate.

On the other hand the validity of Scheme II was further supported by the observation that both oxalacetate decarboxylation and tartarate decomposition were inhibited by dipyridyl (M/340) and malonate (M/40) almost to the same extent (40 to 60%). Anaerobic decomposition of malic acid was also shown to follow Scheme II. From these experimental results it was concluded that this decomposition may proceed via oxalacetate according to the Scheme II.

Aerobic decomposition of tartarate was studied. It was demonstrated that normally a part of tartarate was assimilated while the other part was completely oxidized and no appreciable accumulation of the decomposition products was observed. 2.4 Dinitrophenol inhibited this oxidative assimilation process.

In the normal oxidation of tartarate by the adapted cells a marked lag period was observed in the O_2 uptake and yet CO_2 production started without any appreciable lag. In some instances apparently 1 molecule of net CO_2 production from 1 molecule of tartarate was observed in this early stage without any confusion by the subsequent oxygen uptake. Furthermore, in the presence of monojodacetate O_2 uptake was almost completely inhibited and yet CO_2 production proceeded though at a quite small rate. These observations clearly indicated that the first step of the aerobic decomposition of tartarate is its decarboxylation to the pyruvate stage (possibly via oxalacetate as shown by the other experimental results) and not its dehydrogenation to dihydroxymaleic acid. This conclusion was further supported by the fact that various inhibitors which inhibited pyruvate oxidation also inhibited the oxidation of tartarate. Since DL-glycerate and phosphoglycerate were oxidized only at a very slow rate and the rates were not increased by the adaptation to tartarate, they are unlikely to be the intermediates in the aerobic decomposition, as clearly demonstrated in the anaerobic decomposition.

Monophosphotartarate was decomposed anaerobically by the enzyme preparation which did not show any activity toward tartarate. This result, together with that of the 2.4 dinitrophenol inhibition experiments shown in Fig. 2, suggested the existence of an activation mechanism of tartarate.

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